

Effects of hyperglycaemia in primary human macrophages

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Summary

Background: Patients with type 2 diabetes have a 2-4 fold increased risk of mortality from cardiovascular disease compared to age-matched non-diabetic subjects. This is mainly due to premature atherosclerosis which in part can be accounted for by hyperglycaemia. Early events in human atherosclerosis occur in the intima of the vascular epithelia. Macrophages are the most prominent cell type in atherosclerotic lesions, and uptake of oxidised LDL and accumulation of cholesterol esters in monocyte/macrophages (foam cell formation) is an important early event in atherosclerosis. Previous work in J774.2 murine macrophages exposed to hyperglycaemia in combination with insulin or leptin, show that the rate of cholesterol ester deposition significantly increased through down-regulation of hormone-sensitive lipase (HSL) and up-regulation of acyl-CoA-cholesterol acyltransferase (ACAT). The transcriptional repressor Id2 has the capability to suppress HSL promoter activity, and in J774.2 macrophages Id2 is up-regulated by hyperglycaemia via the hexosamine signalling pathway, involving the rate-limiting enzyme, GFAT, and the enzyme responsible for O-linked glycosylation of proteins, OGT, indicating a direct role of Id2 in macrophage foam cell formation. The role of hyperglycaemia and the hexosamine signalling pathway in primary human macrophages has never been established.

Methods: Peripheral blood mononuclear cells (PBMC) from healthy donors were differentiated for 7 days into macrophages. The macrophages were cultured for different times under normoglycaemic (5 mM) or hyperglycaemic conditions (20 mM) or in the absence or presence of glucosamine, PUGNAc or fructose at 5 mM glucose. Cells were lysed in RIPA buffer and protein samples were separated by SDS-PAGE. GFAT, OGT, Id1 and Id2 protein expression as well as abundance of O-GlcNAcylated proteins were determined by Western blotting, and subcellular localization of OGT, O-GlcNAcylation and Id2 was studied by immunofluorescence.

Results: Fructose and glucosamine induce Id1 and Id2 protein expression as well as the abundance of O-GlcNAcylated proteins in primary human macrophages. Moreover, in accordance with previous observations in the J774.2 murine macrophages cell line, Id2 was found to be a target for O-linked glycosylation in primary human macrophages. Furthermore, we demonstrated nucleo-cytoplasmic shuttling of Id2 and OGT by high

glucose, but in contrast to observations in J774.2 cells, hyperglycaemia has no effect on the protein levels of Id1 and Id2. Finally, hyperglycaemia did not regulate GFAT and OGT protein levels in primary human macrophages.

Conclusion: In resting human macrophages, the hexosamine signalling pathway is involved in the up-regulation of Id1 and Id2 protein, but hyperglycaemia is not able to increase flux through the hexosamine signalling pathway as compared to murine macrophages and does not regulate GFAT and OGT protein levels.

List of abbreviations

PKC	protein kinase C
AGE	advanced Glycated Endproducts
NADPH	nicotinamid adenine dinucleotide phosphate
NO	nitrogen oxide
DAG	diacylglycerol
RAGE	AGE receptor
NF- κ B	nuclear factor κ B
G-6-P	glucose-6-phosphate
F-6-P	fructose-6-phosphate
Glucosamine-6-P	glucosamine-6-phosphate
GlcNAc	nitrogen-acetylglucosamine
UDP	uridine diphosphate
O	oxygene
Ser	serine
Thr	threonine
CoA	acetyl-coenzyme A
GFAT	L-glutamine:D-fructose-6-phosphate amidotransferase
GLUT	glucose transporter
PI3-K	phosphatidyl inosityl-3-kinase
IRS-1	insulin receptor substrate-1
mRNA	messenger Ribonucleic acid
cAMP	cyclic adenosine 3,'5'-monophosphate
PKA	protein kinase A; cAMP dependent protein kinase
OGT	O-linked-N-acetylglucosaminyl (GlcNAc) transferase
OGA	O-GlcNAcase
GlcNAc	N-acetylglucosaminyl
O-GlcNacylation	O-linked glycosylation

kDa	kilodalton
TPR	tetratrcopeptide repeat
ncOGT	nucleocytoplasmic β -N-acetylglucosaminyl transferase
mOGT	mitochondrial β -N-acetylglucosaminyl transferase
PUGNAc	O-(2-acetaminido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate
DON	6-diazo-5-oxo-norleucine
HAT	histone acetyl transferase
NPC	nuclear pore complex
ATPase	adenosine triphosphatease
eNOS	endothelial nitric oxide synthase
Sp1	promoter-specific transcription factor-1
p53	tumor protein 53
Id	inhibitor of differentiation and DNA binding
bHLH	basic helix-loop-helix
L/Z	leucine zipper
PEPCK	phosphoenolpyruvate carboxykinase
SREBP	sterol regulatory element binding protein
HSL	hormone-sensitive lipase
VCAM-1	vascular cell adhesion molecule-1
LDL	low density lipoprotein
M-CSF	macrophage-colony stimulating factor
TNF α	tumor necrosis factor α
IL-12	interleukin-12
PDGF	platelet-derived growth factor
CD36	clusters of differentiation 36
EGF	epidermal growth factor
ACAT	acyl-CoA:cholesterol O-acyltransferase
ob	obesity

PBMC	peripheral blood mononuclear cell
SDS-PAGE	sodium doecyl sulphate polyacrylamide gel electrophoresis
PVDF	immobilon-polyvinylidene flouride
HRP	horse radish peroxide
CTD	C-terminal domain (a part of RNA polymerase II)
IgM	immunoglobulin M
HMPS	hexose monophosphate shunt
NLS	nuclear localization signal
NES	nuclear export signal
LPS	lipopolysaccaride
THP1	human acute monocytic leukaemia cell line

1. Introduction

1.1 General introduction

The consumption of refined sugar has increased considerably during the past several decades. Because it primarily consists of empty calories, replacing more nutritious foods with sugar decreases the intake of vitamins, minerals, essential fatty acids and other beneficial nutrients (1). Refined sugar, sucrose, is hydrolysed by sucrase in the small intestine to fructose and glucose (2), and even if the body has an obligatory requirement for glucose, determined largely by the demands of the brain, high blood glucose levels are associated with both immediate and long-term consequences. For these reasons blood glucose concentration is tightly regulated by homeostatic regulatory systems including insulin secretion stimulated by hyperglycaemia and secretion of counter regulatory hormones like glucagon, epinephrine, cortisol and growth hormones to restore normoglycaemia. The rapid absorption of glucose following consumption of refined sugars and high glycemic index meals challenges these homostatic mechanisms. The acute repeated hyperglycaemia as well as long-term hyperglycaemia may cause overeating, obesity, insulin resistance and eventually type 2 diabetes and atherosclerosis (3).

The deleterious effects of type 2 diabetes are mainly due to the development of vascular pathology in the retina, renal glomerulus, peripheral nerve and arteries that supply the heart, brain and lower extremities (4). The fact that hyperglycaemia is the main cause of microvascular events is well-known, and the mechanisms by which they occur, are thoroughly studied (5, 6). Based on epidemiological studies, hyperglycaemia alone is also a risk factor for cardiovascular disease in patients with diabetes, but the molecular mechanisms of accelerated clotting of arteries that result from diabetes-associated hyperglycaemia, are still not fully understood (7).

Fructose, compared to glucose or sucrose, results in significantly lower insulin responses and serum glucose levels, and when used in moderation, fructose is a safe or even desirable sweetener for patients with diabetes (1). However, the adverse effects of fructose on other aspects of metabolism, like glycosylation of tissue proteins, intracellular accumulation of

sorbitol and oxidative stress might counterbalance its influence on glycaemic control (1). Fructose is transported by the facilitative glucose transporter 5 (Glut 5) that unlike other members of this family (Glut 1-4) is a very poor transporter of glucose and appears to function as a fructose transporter (8). Interestingly, previous studies have shown that Glut 5 is strongly up regulated in macrophages during differentiation, suggesting that these cells become extremely sensitive to increased fructose level (8). The main pathway for fructose metabolism is in the liver, due to the presence of the enzyme fructokinase (2). However, increasing serum fructose concentration results in the conversion of fructose to fructose-6-phosphate by hexokinase in extra-hepatic organs (1) suggesting that fructose, at least in part, is able to mimic the deleterious effects of hyperglycaemia.

1.2 Hyperglycaemia

Hyperglycaemia is defined as a condition where plasma glucose level exceeds the normal range of 3-7 mmol/l (9, 10). In healthy individuals normoglycaemia is restored by the action of insulin, but without sufficient insulin, caused by partial or total failure of the pancreatic insulin production (type 1 diabetes) or insufficient insulin production or resistance to its action (type 2 diabetes) long-term hyperglycaemia is obtained (9). Hyperglycaemic damage is thought to be mediated by four different pathways: The polyol pathway, the protein kinase-C (PKC) pathway, the AGE (Advanced Glycated Endproducts) pathway and the hexosamine pathway (4) (Figure 1.1) Hyperglycaemia increases oxidative stress caused by overproduction of superoxide, and it is suggested that this plays a role in the activation of these four pathways (4).

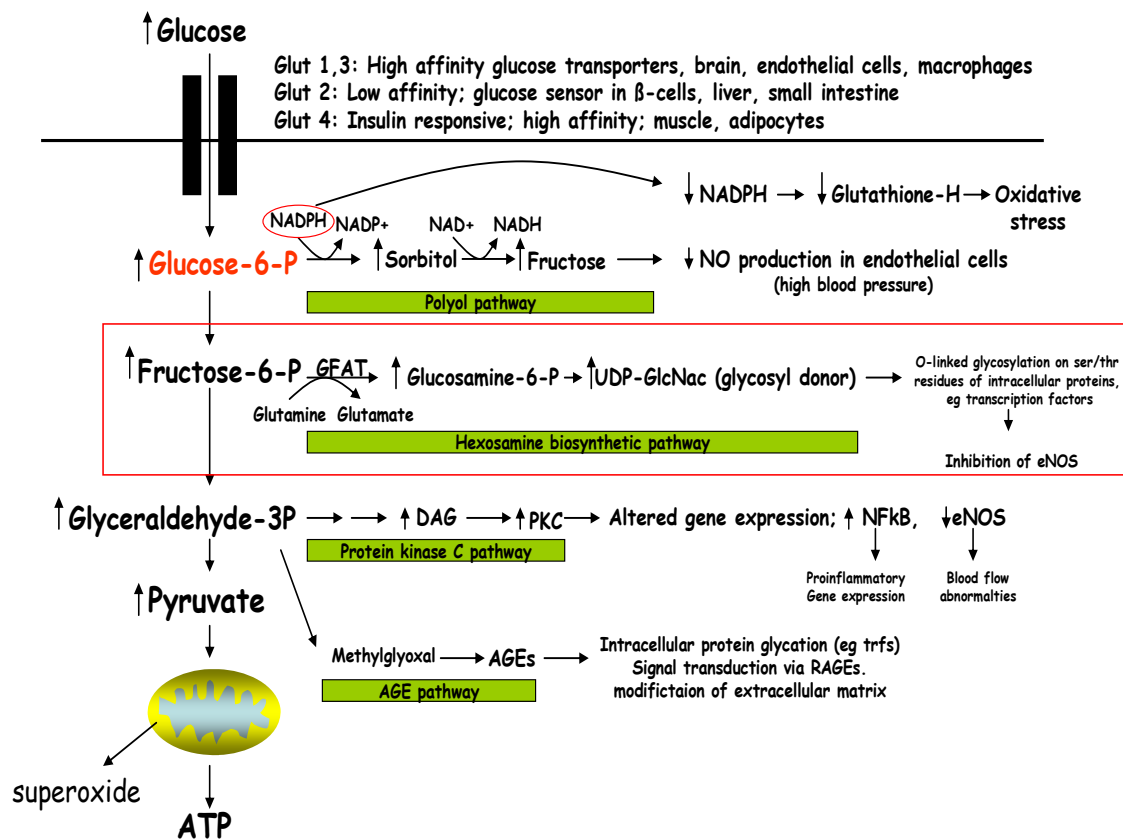


Fig.1.1 Potential mechanism by which hyperglycaemia activates four pathways of hyperglycaemic damage. Increased flux of glyceraldehyde-3-P to DAG, an activator of PKC, and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6-phosphate to UDP-*N*-acetylglucosamine increases modification of proteins by *O*-linked *N*-acetylglucosamine (O-GlcNAc) and increased glucose flux through the polyol pathway consumes NADPH and depletes GSH. (Figure is adapted from Brownlee, 2001 (4)).

1.2.1 Increased polyol pathway flux

The polyol pathway leads to increased intracellular oxidative stress by reducing the amount of the intracellular antioxidant, reduced glutathione (4). The enzyme aldose reductase catalyzes the reduction of glucose to sorbitol using nicotinamid adenine dinucleotide phosphate (NADPH) as cofactor (11, 12). The activity of aldose reductase increases under hyperglycaemic conditions, resulting in consumption of NADPH (4). The recycling of the antioxidant glutathion depends on NADPH supplies, and depletion of these supplies results in intracellular oxidative stress (4, 13, 14). Another reaction that uses NADPH is the synthesis of nitrogen oxide (NO), and if the production of NO is decreased, vasoconstriction and poor blood supply are promoted (15, 16). Additionally, the harmful effects of sorbitol,

includes eye diseases caused by osmotic stress (17) as well as changes in signal transduction and gene expression (18).

1.2.2 Activation of protein kinase C

Hyperglycaemia can mediate its harmful effects by increasing the synthesis of diacylglycerol (DAG), an important activator of α , β , and δ protein kinase-C (PKC), a family of serine-threonine kinases (17). PKC is suggested to play a key role in intracellular signal transduction downstream of hormones (19), and changes in the DAG-PKC pathway can cause alterations in regulatory pathways resulting in functional changes in vascular tissues, leading to endothelial dysfunction (13).

1.2.3 Advanced Glycated End products

In the AGE pathway proteins, lipids and nucleic acids are irreversibly modified by sugar and lipids (20, 21). This nonenzymatic process is initiated by the reaction between a free amino group on a protein or a fatty acid and a carbonyl group on a reduced sugar (22). AGEs were originally thought to arise from reactions between extracellular proteins and glucose, but it is now believed that intracellular hyperglycaemia is the primary event in the formation of both intracellular and extracellular AGEs (4). Reversible Schiff-bases are formed and further transformed into Amadori products, which rearranges to the highly reactive AGE precursors such as glyoxals, methylglyoxals and 3-deoxyglucosone which react with amino groups and lead to the formation of AGE (22). Additionally, AGEs can be formed from other carbohydrates like fructose, ribose or glyceraldehyde or from auto-oxidative glycation (23). Intracellular AGE precursors are able to modify intracellular proteins, including transcription factors, leading to altered cellular functions. Furthermore, AGE precursors may diffuse out of the cell and modify extracellular matrix components. These modified proteins interact abnormally with other matrix components and integrins on cells, and cause cellular dysfunction. Additionally, plasma proteins modified by AGE precursors bind to AGE receptors (RAGEs) on macrophages, endothelial cells and mesangial cells, and this ligation has been shown to activate nuclear factor κ B (NF- κ B) causing pathological changes in gene expression (4).

1.3 The hexosamine signalling pathway

Up to 99 % of intracellular glucose is destined to glycolysis and glycogen storage, but a small fraction (1-5 %) is diverted to the hexosamine pathway (24). This pathway is currently envisioned to consist of two parts; synthesis of uridine diphosphate (UDP) –N-acetylglucosamine (UDP-GlcNAc) and transfer and removal of O-GlcNAc (25). The precursors of the synthesis of UDP-GlcNAc include the amino acid glutamine, lipid derived acetyl-coenzyme A (CoA), glucose and uridine (25). The first part of the pathway is initiated by the conversion of fructose-6-phosphate to glucosamine-6-phosphate by the rate limiting enzyme L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) and culminates in UDP-GlcNAc formation (26, 27, 28). One of the roles of UDP-GlcNAc is to serve as a precursor for the essential formation of complex membranous and secretory glycoproteins in the endoplasmatic reticulum and Golgi apparatus (29, 30), and the hexosamine pathway was previously thought to be entirely a biosynthetic pathway. In 1991, however, new insights into the functional roles of this pathway were obtained (31). Incubation of isolated adipocytes under hyperglycaemic conditions enhanced the flux through the hexosamine pathway and culminated in a state of cellular insulin resistance (31). Insulin resistance is defined as the inhibition of intracellular signal molecules mediating insulin effects, such as glucose transport (9). It occurred that the hexosamine pathway contained a nutrient sensing and metabolic signalling ‘arm’, responsible for desensitizing of the glucose transport system. With this realization, the name ‘Hexosamine signalling pathway’ was proposed (32), a name which from now on is adapted in this particular study. The metabolic effects of this pathway are mediated by O-GlcNAcylation on serine and threonine residues on regulatory proteins, using UDP-GlcNAc as substrate (33, 34) (Figure 1.2). In insulin responsive tissues, like adipose tissue and skeletal muscle, insulin signalling results in facilitative Glut 4 translocation to the cell membrane followed by glucose uptake, and this is dependent on activation of phosphatidyl inositol-3- kinase (PI3-K). (35). In models of type II diabetes, the recruitment of Glut 4 is insufficient, and this is postulated to be caused by a specific defect in PI3-K signalling (36). Also, PI3-K has been shown to be a target for O-linked glycosylation (25). **To elucidate the role of the hexosamine signalling pathway and O-GlcNAcylation in macrophage foam cell formation in atherosclerosis, one specific aim of this study is to investigate whether high glucose via the hexosamine signalling**

pathway increases the abundance of O-GlcNAcylated proteins in primary human macrophages.

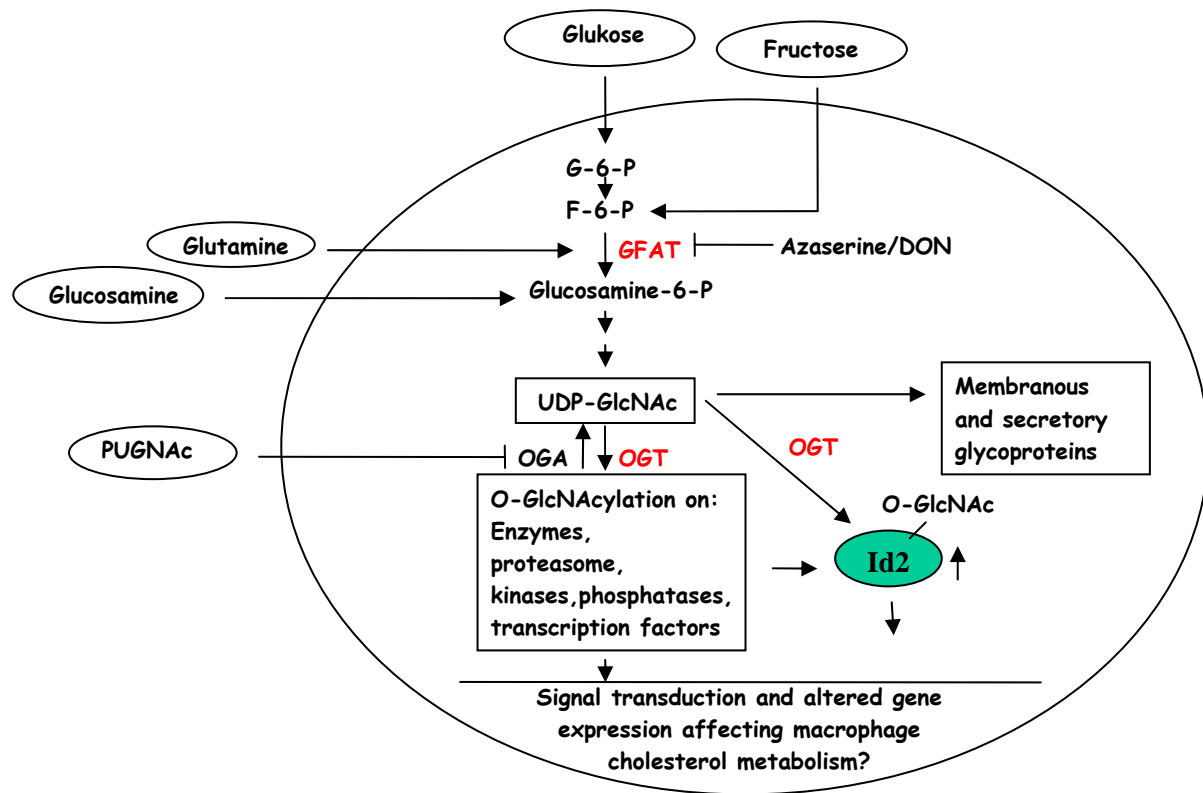


Fig.1.2 The hexosamine signalling pathway. This pathway consists of two parts; synthesis of uridine diphosphate (UDP) – N-acetylglucosamine (UDP-GlcNAc), initiated by the conversion of fructose-6-phosphate (F-6-P) to glucosamine-6-phosphate (glucosamine-6-P) by the rate limiting enzyme L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT), and transfer and removal of O-GlcNAc. (6-diazo-5-oxo-norleucine) DON and azaserine inhibit the enzymatic activity of GFAT. UDP-GlcNAc is precursor for formation of complex membranous and secretory glycoproteins and substrate for O-linked glycosylation (O-GlcNAc). The enzymes OGT and OGA catalyze the turnover of O-GlcNAc, and the enzymatic activity of OGA can be inhibited by O-(2-acetaminido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc). Hyperglycemia and glucosamine increase the flux through the hexosamine biosynthetic pathway, leading to an increase in the abundance of O-GlcNAc modifications. As serum concentration of fructose increases, fructose is converted to fructose-6-phosphate by hexokinase in extra-hepatic organs, and is also likely to increase the flux through this pathway. O-GlcNAc modifications lead to functional changes in target proteins (such as the transcriptional repressor Id2) and can affect transcription, translation and signalling.

1.3.1 GFAT

The rate-limiting enzyme of the hexosamine signalling pathway is L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) (26, 27, 28). GFAT is a 681 amino-acid protein with a molecular weight of approximately 77 kDa (28) and composed of two domains: a glutaminase domain, which catalyzes the hydrolysis of glutamine to glutamate and ammonia, and a synthase domain, which catalyzes the amination and isomerization of fructose-6-phosphate to glucosamine-6-phosphate (37). As GFAT is rate limiting in the synthesis of glucosamine, the main substrate for protein glycosylation, the quantity and activity of this enzyme is critical for the essential glycoprotein synthesis in eucaryotic cells (38). There are two isozymes of GFAT; GFAT1 and GFAT2. They are encoded by different genes and their relative expression varies among tissues; GFAT1 is ubiquitous with high levels of expression of protein and mRNA in adipocytes and skeletal muscle, and a marked, but varying expression in vascular smooth muscle cells (39). GFAT2 is preferentially expressed throughout the central nervous system, especially in the spinal cord (40).

Several studies show that GFAT is under very tight regulation (38, 39). In adipocytes the enzyme activity is inhibited by glucose and insulin (41, 42), and in all eukaryotes, UDP-GlcNAc allosterically inhibits the activity (28, 43). Additionally, phosphorylation by cAMP-dependent protein kinase A (PKA) appears to act as a modulator of GFAT activity (27). However, expression of GFAT protein in skeletal muscle seem to be increased when plasma concentration of the saturated fatty acids palmitate and stearate are high (44). The fact that GFAT is highly expressed in most tissues involved in the development of diabetic late complications indicates that GFAT expression may be induced by manifest diabetes (39). **GFAT protein expression in primary human macrophages has not previously been studied, and one particular aim of this study is to investigate whether high levels of glucose, via the hexosamine signalling pathway, affects GFAT protein expression.**

1.3.2 O-linked glycosylation, OGT and OGA

O-linked glycosylation of serine and threonine residues of various regulatory proteins is a reversible protein modification (45). O-GlcNAc is ubiquitous and essential, and so far, more than 500 proteins, involved in almost all aspects of cellular processes, have been identified to be O-GlcNAcylated (24, 25, 46). Unlike the static nature of extra cellular glycosylation,

O-GlcNAc cycles dynamically in response to the extracellular environment (35). O-GlcNAcylation appears to be as abundant as phosphorylation (33, 34), and occurs both simultaneously and reciprocally to phosphorylation (47) (Figure 1.3). There is a complex and dynamic interplay between O-GlcNAc and O-phosphate, and the combination of modifications creates enormous molecular diversity to fine tune protein interactions and functions (48). Concentrations of the substrate for O-GlcNAcylation, UDP-GlcNAc, are highly sensitive to fluxes in nutrients, energy and metabolic nitrogen, and rapid changes in UDP-GlcNAc concentration serves as a sensor by directly affecting the extent of O-GlcNAcylation and thus, phosphorylation of regulatory proteins (48). The enzymes catalyzing the addition and removal of O-GlcNAc has been characterized, and unlike protein phosphorylation in which approximately 650 enzymes are involved in the regulation, only two catalytic enzymes, OGT and OGA, catalyze the turnover of O-GlcNAc (35).

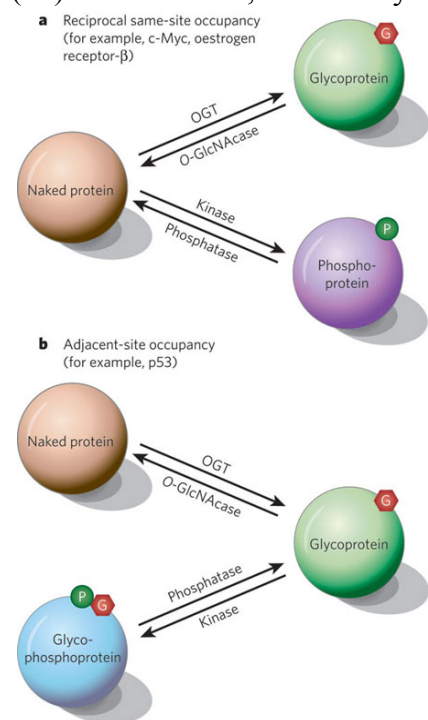
OGT

The enzyme responsible for the transfer of UDP-GlcNAc to serine and threonine protein residues is O-linked N-acetylglucosaminyl (O-GlcNAc) transferase (OGT) (45). OGT is a heterotrimer composed of two catalytic 110-kDa subunits and one regulatory 78-kDa subunit (49). The molecule is encoded by a single gene on the X chromosome (45), and gene knock-out experiments have shown that OGT is essential for stem cell viability and embryonic development (50). OGT consists of an N-terminal segment with targeting information, a tetratrapeptide repeat (TPR) domain, a linker region and a C-terminal highly conserved catalytic domain (25). TPRs are 34-amino acid repeats that facilitate protein-protein interactions (25). The OGT isoforms contain between 1 to more than 16 TPRs, and varying the number of TPR domains affect the substrate recognition, trimerization (51) and cellular localization (45, 52, 53) of the enzyme. In mammalian cells two transcripts are well characterized; nucleocytoplasmic OGT (ncOGT) and mitochondrial OGT (mOGT) (34, 54, 55). As OGT seem to be predominantly located in the nucleus, the nucleocytoplasmic OGT (ncOGT) is the most thoroughly studied of the known splice variants (34). OGT is subject to autoglycosylation and tyrosine phosphorylation (56), suggesting that these post-translational modifications are regulators of the enzymatic activity. Furthermore, the concentration of UDP-GlcNAc, which increases in response to hyperglycaemia, has also been shown to play a key role in modulating the activity of OGT (35). The K_m of OGT for different substrates changes at different UDP-GlcNAc concentrations (57), and as UDP-GlcNAc rapidly changes

in concentration in response to nutrients and environmental factors, OGT is able to sense these changes and in turn, transduce this information to protein regulatory networks (46). In liver cells, insulin stimulates the synthesis of OGT and enhances cytosolic staining of OGT (58). **The OGT protein expression in primary human macrophages has not yet been studied. One specific aim of the current study is to study the subcellular distribution of OGT as well as the regulation of OGT protein expression by high glucose and the hexosamine signalling pathway.**

OGA

Removal of O-GlcNAc is performed by a ubiquitous, nuclear/cytosolic β -N-acetylglucosaminidase called O-GlcNAcase (OGA) (50). This is a 917 amino-acid heterodimer composed of a 54-kDa α -subunit and a 51-kDa β -subunit. (50, 59, 60, 61). The enzymatic activity of OGA can be inhibited by O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc). PUGNAc is an O-GlcNAc analogue that prevents cycling of O-GlcNAc on proteins without significantly altering N-linked glycosylation or UDP-GlcNAc levels (63). OGT and OGA are strongly associated into a single complex (64). Experiments in transgenic mice have shown that this complex is essential for proper estragon dependent cell signalling as well as mammary development (64). Furthermore, these enzymes are components of several multiple factor holoenzyme



complexes which appear to regulate both the targeting and activity of O-GlcNAc cycling (45, 52, 53, 65, 66).

Fig.1.3 There is a complex and dynamic interplay between O-GlcNAc and O-phosphate. On some proteins O-GlcNAc and O-phosphate compete dynamically for the same serine or threonine hydroxyl moiety. This reciprocal occupancy seems to produce different activities or stability in the proteins (a). In certain proteins, O-GlcNAc and O-phosphate can occur next to each other. Adjacent occupancy by each modification reciprocally influences the functions or turnover of proteins (b) (Figure is adapted from Hart *et al.*, 2007 (48)).

O-GlcNAcylated proteins

Several cytosolic and nuclear proteins have been identified as targets for O-GlcNAylation including kinases, phosphatases, transcription factors, metabolic enzymes, chaperones and cytoskeletal proteins (25). The O-linked glycosylated nuclear pore protein p62 is crucial for the nuclear pore complex (NPC) formation and directly involved in the nuclear transport process (67). Furthermore, O-GlcNAc modification of the 26S proteasome prevents proteolysis of ubiquitinated transcription factors like Sp1 by inhibiting the adenosine triphosphatase activity (ATPase) activity of the proteasome (68). Another example, in which O-GlcNAcylation inhibits substrate activity, is by inhibition of the enzyme eNOS (25). The Akt phosphorylation site of eNOS seems to be glycosylated instead of phosphorylated under hyperglycaemic conditions, and this O-GlcNAc modification and impairment of eNOS contributes to endothelial dysfunction in atherosclerosis (69).

Furthermore, some cytosolic proteins need to be in their O-GlcNAc state to be transported to the nucleus. One of these proteins is Tau, which belongs to the family of brain microtubule-associated proteins. Tau protein is O-linked glycosylated as well as phosphorylated, and it seems like the nuclear localisation is regulated by relative abundance of these two modifications. (70)

Transcription factors make up most of the O-GlcNAc modified proteins (71). The first identified was Sp1, a regulator of several house keeping genes. It has multiple O-GlcNAc modification sites, and its phosphorylation on Ser/Thr is inversely proportional to its O-GlcNAc modification. (33). cMyc regulates gene transcription in cell proliferation, cell differentiation and programmed cell death, and both O-GlcNAcylation of Sp1 and cMyc increases their transactivating potential (47). O-GlcNAc modification also has a protective role during stress responses (25). Increased O-linked glycosylation of the tumour suppressor p53, in response to cellular stress, including DNA damage, is stabilizing the protein and increases its anti-proliferative activity (46, 47, 72).

1.4 Inhibitor of DNA binding (Id) family

Inhibitor of differentiation and DNA binding (Id) genes encode a family of 4 basic helix-loop-helix (bHLH) proteins (Id1, Id2, Id3 and Id4) that lack the basic DNA binding domain

(73). Id proteins are able to act as transcriptional repressors by dimerizing with the class A bHLH transcription factors (E-proteins) and form non-functional heterodimers. Thus, Id proteins act as dominant-negative regulator of these transcription factors and have the capability to modulate a wide range of gene expression events in the cell (74, 75, 76). Id1, Id2 and Id3 are expressed ubiquitously, and Id4 is expressed predominantly in testis, brain and kidney. They act as positive regulators of cell proliferation, and their gene expression is highest in proliferating cells (73). Id mRNA concentrations rapidly increases in response to serum or growth factors, including insulin (77), and previous studies have shown that Id1 protein is regulated by glucose (77) and fatty acids (78) in β -cells. Recently, in a murine cell line, Id2 protein expression was shown to be up-regulated by glucose (79). Furthermore, overexpression of GFAT in J774.2 macrophages increased Id2 protein levels, suggesting that the hexosamine signalling pathway is mediating the effect of high glucose on Id2 (unpublished data). Id2 is targeted to proteasomal degradation through N-terminal ubiquitination by 26S (80), and O-GlcNAc modification of the 26S proteasome inhibits proteolysis through inhibition of the ATPase activity of the proteasome (68). This may be a mechanism by which hyperglycaemia via the hexosamine signalling pathway increase Id2 protein expression in macrophages. Moreover, experiments in J774.2 macrophages indicate that Id2 itself is a target for O-GlcNAcylation. This may lead to stabilization of the protein by making it less susceptible to proteasomal degradation (unpublished data). **One aim of the current study is to verify the O-GlcNAcylation of Id2 in primary human macrophages.**

Experiments in hepatocytes show that overexpression of GFAT causes reduced hepatic gluconeogenesis compared to controls despite of lower fasting glucose and insulin levels (26). Furthermore, overexpression of Id2 in hepatocytes suppresses Dexametasone/cAMP-induced activation of phosphoenolpyruvate carboxykinase (PEPCK) promoter causing inhibition of hepatic gluconeogenesis, suggesting a role for the hexosamine signalling pathway and Id2 in mediating the inhibiting effect of glucose on hepatic glucose production (unpublished data)(Figure 1.4).

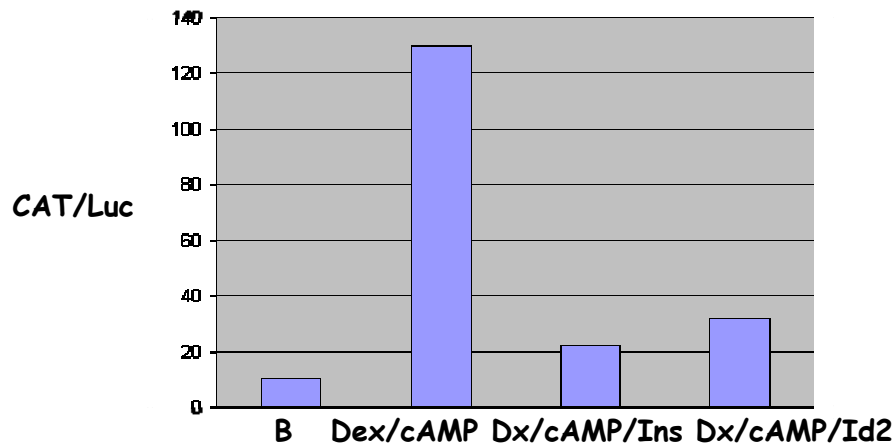


Fig.1.4 Overexpression of Id inhibits Dexametasone/cAMP-mediated activation of PEPCK gene expression in H4IIE hepatoma cells. Overexpression of Id2 in hepatocytes suppresses Dexametasone/cAMP-induced activation of phosphoenolpyruvate carboxykinase (PEPCK) promoter causing inhibition of hepatic gluconeogenesis.

Growing evidence suggest that increases in Id2 may have functional effects on metabolic genes (79). The bHLH-L/Z transcription factor SREBP is essential for adipocyte differentiation and the expression of genes controlling lipid metabolism (74), and Id2 has been shown to bind to and inhibit the activity of SREBP-1c (74). SREBP-1 stimulates the promoter of the cholesterol ester hydrolase (hormone-sensitive lipase; HSL), the enzyme responsible for cholesterol ester hydrolysis in macrophages (79). Furthermore, experiments in J774.2 cells shows that Id2 antagonizes the stimulatory effects of SREBP-1, and down-regulates SREBP-1 mediated induction of HSL promoter activity, suggesting a role for Id2 in glucose-mediated cholesterol ester deposition in macrophages (79, 81) (see below).

1.5 Hyperglycaemia and cardiovascular disease

Early events in atherosclerosis involve adhesion of circulating monocytes to the vascular endothelium, followed by transmigration of monocytes into the subendothelial space (82).

In response to the secretion of macrophage-colony stimulating factor (M-CSF) by activated smooth muscle cells and endothelial cells, monocytes differentiate into macrophages (83). Macrophages express scavenger receptors for modified lipoproteins, such as oxidized LDL, and ox-LDL accumulate large amount of cholesterol ester, resulting in the formation of lipid-laden foam cells (83, 84). Both the process of monocyte transmigration and macrophage CE accumulation is increased under hyperglycaemic conditions. The effect of hyperglycaemia is, at least in part, due to increased expression of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemo attractant protein-1 (MCP-1) on endothelial cells, mediated by intracellular stress and activation of NF- κ B (85, 86) (Figure 1.5)

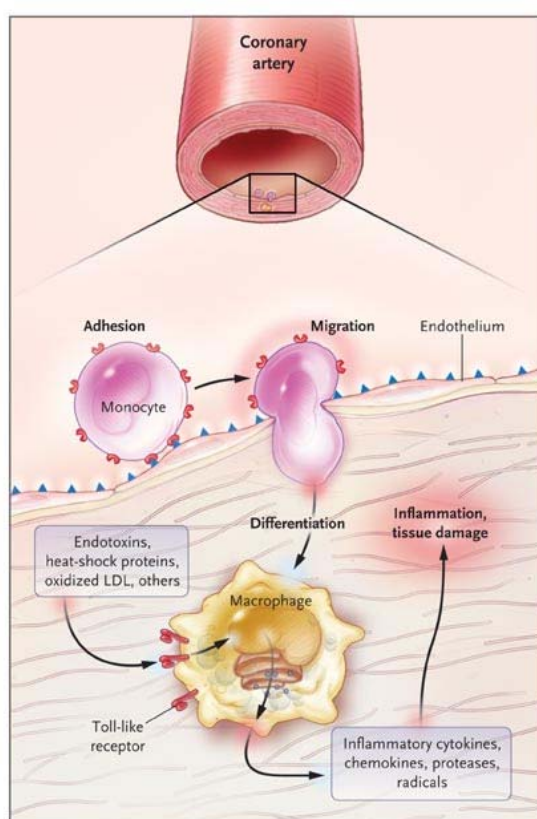


Fig.1.5 Role of macrophage inflammation of the artery. Adhesion of circulating monocytes to the vascular endothelium, and transmigration of monocytes into the subendothelial space, followed by differentiation into macrophages, and ultimately, to inflammation and tissue damage (Figure is adapted from Hansson GK, 2005 (87)).

It is now well established that growth factors and cytokines can act to promote atherogenesis. However, little is currently known about how cholesterol ester metabolism in macrophages is affected by the conditions that characterize type-2 diabetes, insulin resistance, and obesity such as hyperglycaemia, hyperleptinemia and hyperinsulinemia. In murine J774.2 macrophages chronic exposure to hyperglycaemia increases the uptake of ox-LDL by up-regulation of scavenger receptor CD36 with no net increase in cholesterol ester

accumulation (81). When these cells were exposed to hyperglycaemia in combination with insulin or leptin, the rate of cholesterol ester deposition increased significantly through up-regulation of the activity of the main enzyme responsible for cholesterol ester synthesis, acyl-CoA:cholesterol O-acyltransferase, (ACAT) and down-regulation of HSL protein expression and activity (81).

As previously explained, Id2 is likely to mediate down-regulation of HSL promoter activity (79). Interestingly, Id2 is also one of the genes found to be over expressed in muscle, fat and liver of ob/ob mice (79). These mice are a genetic model of obesity and severe insulin resistance (88). Furthermore, preliminary data indicates that Id2 knock out mice are resistant to development of atherosclerosis suggesting that Id2 contributes significantly to the atherogenic process.

1.6 Aim of study

Previous work in J774.2 murine macrophages shows that Id2 is up-regulated by hyperglycaemia via the hexosamine signalling pathway, involving the rate-limiting enzyme, GFAT, and the enzyme responsible for O-linked glycosylation of proteins, OGT. When J774.2 cells are exposed to hyperglycaemia in combination with insulin or leptin, the rate of cholesterol ester deposition increase through up-regulation of ACAT and down-regulation of HSL. Overexpression of Id2 is able to suppress HSL promoter activity in J774.2 cells, suggesting a role of Id2 in glucose-mediated cholesterol ester deposition. The role of hyperglycaemia and hexosamine signalling pathway in human macrophages has not yet been studied, and specific aims of this study in *primary human macrophages* are to:

- investigate high glucose/hexosamine-mediated regulation of Id2 and Id1 protein expression
- study the effect of hyperglycaemia/hexosamine on subcellular distribution of Id2
- study hyperglycaemia/hexosamine-induced O-GlcNAcylation of Id2
- investigate high glucose/hexosamine-mediated regulation of GFAT protein expression
- investigate high glucose/hexosamine-mediated regulation of OGT protein expression

- study the subcellular distribution of OGT and O-GlcNAcylated proteins in normoglycaemic versus hyperglycaemic cells
- identify novel hexosamine-induced O-GlcNAcylated proteins

Due to the observation that the fructose transporter Glut 5 is strongly up-regulated in human macrophages during differentiation, one specific aim is to:

- investigate whether fructose induces the protein expression of Id1, Id2, GFAT and OGT and whether fructose affects the abundance of O-GlcNAcylated proteins in primary human macrophages

2. Methods

2.1 Isolation of human monocytes

Peripheral blood mononuclear cells (PBMC) can be isolated from whole blood using different density gradient centrifugation procedures. Anticoagulated whole blood is layered over the separation medium, and at the end of the centrifugation step, the following layers are visual (from top to bottom): Plasma/platelets, PBMC, separating medium and erythrocytes and granulocytes. The PBMC layer is then collected and washed to get rid of contaminants before cell type and cell viability can be confirmed.

Materials:

- Anticoagulated whole blood (Ullevål Hospital, Norway)
- Lymphoprep (Fresenius Kabi Norge AS, Norway)
- PBS (free of LPS, without Ca^{2+} and Mg^{2+}) (Gibco; Invitrogen, Norway; cat.no: 10010-015)
- RPMI 1640 medium/ GlutaMAXTM-1 (Gibco; Invitrogen, Norway; cat.no: 61870)
- Human AB serum (Bio WhittakerTM; cat.no: 14-490E)
- M-CSF (PEPROTECH EC, UK; cat.no: 300-25)
- Penicillin/ Streptomycin (Gibco; Invitrogen, Norway; cat.no: 15140-122)
- 10 cm cell culture plates (NUNCTM, Denmark; cat.no: 15140-122)
- Centrifuge with cooler (Jouan CR412, USA)
- Cell counter (Beckman coulterTM Z2 coulter^R Particle count and size Analyzer, Nerliens Meszansky AS, Norway)
- CO₂ Air-Jackeded Incubator DH Autoflow (NUAIRETM, UK)

Procedure:

Buffy coats were obtained from healthy donors at the Ullevål hospital blood bank. The contents of the buffy coat were transferred to 5 falcon tubes (50 ml), and 35 ml PBS (free of LPS with no calcium or magnesium) was added to each tube. The samples were mixed by pipetting, and then 10 ml lymphoprep was carefully added to the bottom of each tube. The

samples were then spun at 500 x g for 30 min at room temperature, with no brake. The cells recovered from the interface were transferred into 2 new falcon tubes. Cells were washed two times by adding PBS up to 50 ml, spinning the tubes at room temperature for 10 min at 500 x g and discarding the supernatant. Cell pellets were then resuspended in PBS and transferred to one tube. PBS was added to 50 ml, and the tube was spun for 5 min at 300 x g at room temperature. Cell pellet was then resuspended in 10 ml RPMI 1640 medium/GlutaMAXTM-1 and the cells were counted using Beckman CoulterTM Particle count and size Analyzer. RPMI 1640 medium/GlutaMAXTM-1 was added to achieve the concentration of 2.5-3 mill. cells/ml. 10 % human AB serum was added, and the cell solution were transferred to 10 cm cell culture plates, 10 ml to each dish. The cells were allowed to adhere to plastic for 1 hour at 37°C in a humidified 5 % CO₂ atmosphere before the medium was removed, and the cells were washed extensively 3 x in PBS in order to remove contaminant cells. 10 ml RPMI 1640 medium containing 5 % human AB serum, 25 ng/ml macrophage-colony stimulating factor (M-CSF) and 1 % penicillin/ streptomycin were added to the remaining adherent monocytes, and the monocytes were incubated for 7 days at 37°C in humidified 5 % CO₂ atmosphere to differentiate to macrophages.

2.2 Stimulation of cells

Materials:

- PBS (without Ca²⁺ and Mg²⁺) (Gibco; Invitrogen, Norway; cat.no: 10010-015)
- D-glucose medium: (RPMI 1640 (without glucose) (Gibco; Invitrogen, Norway; cat.no: 11879-020), D-Glucose (Sigma-Aldrich Co, USA; cat.no: G 6152))
- D-(+)-glucosamine hydrochloride (Sigma-aldrich Co, USA; cat.no: G 4875)
- D-fructose (Sigma-Aldrich Co, USA; cat.no: F 2543)
- O-(-2-acetamido-2-deoxy-D-glucopyranosylidene amino N-phenylcarbamate)
(PUGNAc) (Toronto Chemical Inc)
- Insulin (from bovine pancreas) (Sigma-Aldrich Co, USA; cat.no: I-6634)
- Leptin (from mouse) (AbD Serotec, UK; cat.no: PMP 25)

Procedure:

After 7 days in culture, the macrophages adhered to the plates were washed 2 x with 10 ml PBS. The cells were then incubated over night at 37°C in humidified 5 % CO₂ atmosphere in RPMI 1640 medium (with L-glutamine, without glucose) containing 1 % human AB serum and 0.5 mM or 5 mM D-glucose. The next day the cells were stimulated with various glucose concentrations or in the absence or presence of insulin and/or leptin, glucosamine, PUGNAc or fructose in RPMI 1640 medium (with L-glutamine, without glucose) containing 1 % human AB serum and 0.5 mM, 5 mM or 20 mM D-glucose. Stimulation with glucosamine, PUGNAc and fructose were only performed in normoglycaemic conditions (5 mM glucose).

2.3 Cell lysate preparation**Materials:**

- PBS (without Ca²⁺ and Mg²⁺) (Gibco; Invitrogen, Norway, cat.no: 10010-015)
- Cell scrapers (3008) (Costar^R, USA; cat.no: SPD 273384)
- Centrifuge with cooler (Centrifuge 5415R, Eppendorf GmbH, Germany)
- Heating block (Ori-Block 08-1, Techne)
- 1 x Loading buffer:
 - Glycerol (Sigma-Aldrich Co, USA; cat.no: G 5516)
 - SDS (Sigma-Aldrich Co, USA; cat.no: L 4509)
 - DTT (Fermentas, Sweden; cat. no: R 0861)
 - Bromophenol blue (Sigma-Aldrich Co, USA; cat.no: B 0126)
 - Tris/HCl (Sigma-Aldrich Co, USA; cat.no: T 1503)
- 1 x RIPA-buffer:
 - (Tris/HCl (Sigma-Aldrich Co, USA; cat.no: T 1503)
 - NaCl (Fluka Chemicals GmbH, Germany; cat.no: 71381)
 - Sodium dodecyl sulfate (SDS) (Sigma-Aldrich Co, USA; cat.no: L 4509)
 - NP-40 (BioVision, USA; cat.no: 2111-100)
 - Na- Deoxycholate (Sigma-Aldrich Co, USA; cat.no: D 6750)

-
- EDTA (Sigma-Aldrich Co, USA; cat.no: E 5134)
 - Tx-100 (Sigma-Aldrich Co, USA; cat.no: X 100)
 - ALLN (Calbiochem^R, USA; cat.no: 208719)
 - AEBSF (Calbiochem^R; USA: cat.no: 101500)
 - Mix of protease inhibitors (Roche Diagnostics, Germany; cat.no: 13181300)
 - DTT (Fermentas, Sweden; cat.no: R 0861)
 - NaF (Sigma-Aldrich Co, USA; cat.no: S 6776)
 - Na₄PO₂O₇ (Sigma-Aldrich Co, USA; cat.no: S 9515)
 - N₃VO₄ (Sigma-Aldrich Co, USA; cat.no: S 6508)

Procedure:

The plates were placed on ice, and the cell monolayers were washed 3 x in 10 ml ice-cold PBS carefully added to one side of the plate and removed by suction. After removal of PBS, 200 µl RIPA buffer were added to the cell monolayer in order to lyse the cells. The lysates were then scraped of the plate and transferred to ice-cold eppendorf tubes. The tubes were incubated on ice for 30 min and insoluble fraction was removed from the lysates by centrifugation at 14 000 rpm for 10 min at 4°C. The supernatants were then transferred to new, ice-cold eppendorf tubes and 20 µl was removed for protein concentration measurements. 50 µl 5 x Loading Buffer was added to 200 µl lysate and the samples were boiled for 5 min.

2.4 Measurement of protein concentration

The Bio-Rad Protein Assay, based on the method of Bradford, is an accurate procedure for determining concentration of solubilised protein. Assay Dye Reagent Concentrate is added to a protein sample and differential colour change of the dye occurs in response to various concentration of the protein

Materials:

- Albumine from bovine serum (BSA) (Sigma-Aldrich Co, USA; cat.no: A 9418)
- RIPA buffer (as described)

-
- Bio-Rad Protein Assay Dye Reagent (Bio-Rad™, USA; cat.no: 500-0006)
 - Cyvettes (Chemi-Technik as, Norway)
 - Spectrophotometer (Ultrospec 3000, UV/visible spectrophotometer; Pharmacia Biotech)

Preparation of a standard curve:

A standard curve using different volumes of 1 µg/µl BSA was prepared in order to provide a relative measurement of the protein concentrations. MilliQ H₂O, BSA (1 µg/µl) and RIPA buffer were transferred to eppendorf tubes as follows:

Mg	BSA (1 µg/µl) (µl)	Buffer	dH ₂ O (µl)
1	1.0	4.0	795
2	2.0	4.0	790
4	4.0	4.0	790
6	6.0	4.0	790
8	8.0	4.0	790
10	10.0	4.0	785
Blank	0.0	4.0	795

200 µl Bio-Rad Protein Assay Dye Reagent was then added to each tube, and the tubes were mixed by vortexing. The samples were incubated for 5 min at room temperature and then transferred to cyvettes. The cyvettes were placed in the spectrophotometer, and a standard curve was then detected from the different volumes of BSA.

Protein measurements of lysates:

2 parallels of each lysate were prepared in eppendorf tubes by adding 795 µl milliQ H₂O, 2 µl RIPA buffer and 2 µl of the lysates. 200 µl Bio-Rad Protein Assay Dye Reagent was added to each tube, and the tubes were mixed by vortexing. The contents of each tube were then added to cyvettes and the cyvettes were placed in the spectrophotometer. Measurements at 595 nm were performed and the relative protein concentrations of each lysate compared to the standard curve were then decided.

2.5 SDS polyacrylamid gel electrophoresis

In order to visualize and separate protein, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. SDS is an anionic detergent and a polypeptide chain binds amounts of SDS in proportions to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted to an anode in an electric field.

Materials:

- CriterionTMPrecast gel (7.5 %) (Bio-RadTM, USA; cat.no: 345-005)
- CriterionTMPrecast gel (10 %) (Bio-RadTM, USA; cat.no: 345-009)
- CriterionTMPrecast gel (4-15 %) (Bio-RadTM, USA; cat.no: 345-0027)
- CriterionTMPrecast gel (15 %) (Bio-RadTM, USA; cat.no: 345-0019)
- 1 x Running buffer
 - Tris/ HCl (Sigma-Aldrich Co, USA; cat.no: T 1503)
 - Glycine (Sigma-Aldrich Co, USA; cat.no: G 7126)
 - SDS (Sigma-Aldrich Co, USA; cat.no: L 4509)
- Precision Plus Protein Standards (All blue) (Bio-RadTM, USA; cat.no: 161-0373)
- Precision Plus Protein Standards (Dual colour) (Bio-RadTM, USA; cat.no: 161-0374)
- BIORAD Criterion System (Bio-RadTM, USA)

Procedure:

CriterionTMPrecast gel was transferred to the BIORAD Criterion System and the chamber was filled with 1 x Running buffer. The same amount of protein (~ 35 µg) as well as Precision Plus Protein Standards (All blue and Dual colour) was carefully loaded to each well. The lid was placed on the chamber and electrophoresis was performed under constant voltage of 150 V for about 90 min.

2.6 Wet transfer and western blot

Samples were transferred from polyacrylamide gels to Immobilon-Polyvinylidene fluoride (PVDF) blotting membrane using BIORAD Criterion blotter system.

Materials:

- Immobilon-Polyvinylidene fluoride (PVDF) blotting membrane (Millipore, Norway; cat.no: IPVH00010)
- GFAT (Nerlich, 1998 (39))
- OGT/AL28 (Hart, 2003 (45))
- Id1 (c-20) rabbit polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz; cat.no: Sc-488)
- Id2 (c-20) rabbit polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz; cat.no: Sc-489)
- CTD 110.6 monoclonal antibody (Covance, UK, cat.no: MMS-248R)
- RL-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz; cat.no: Sc-59624)
- Goat Anti-Rabbit IgG secondary antibody (Jackson immuno research, USA; cat.no: 111-035-144)
- Goat Anti-Mouse IgM Secondary antibody (Sigma-Aldrich Co, USA; cat.no: A 8786)
- 10 x Towbin buffer:
 - Tris/HCl (Sigma-Aldrich Co, USA; cat.no: T 1503)
 - Glycine (Sigma-Aldrich Co, USA; cat.no: G 7126)
 - Methanol (BDH-Prolab, UK; cat.no: UN-1230)
- 1 x TBS-T:
 - Tris/HCl (Sigma-Aldrich Co, USA; cat.no: T 1503)
 - NaCl (Fluka, Germany; cat.no: 71381)
 - Tween 20 (Sigma-Aldrich Co, USA; cat.no: P 9416)
- 3 % Albumine from bovine serum (BSA) (Sigma-Aldrich Co, USA; cat.no: A 9418) in TBS-T
- Fibre pads (Bio-RadTM, USA)
- Filter papers (Bio-RadTM, USA)
- Methanol (BDH-Prolab, UK; cat.no: UN-1230)
- Ponceu (0.1 %)(v/v) in 5 % acetic acid (v/v)(Sigma-Aldrich Co, USA; cat.no: P 7170)
- Supersignal West Pico Chemiluminescent substrate (PIERCE, USA; cat.no: 34080)

-
- ECL Supersignal West Dura extended Duration Substrate (PIERCE, USA; cat.no: 34076)
 - Rocking platform (Platform STR8, Stuart Scientific, UK)
 - Hypercassette (Amersham Biosciences, UK)
 - HyperfilmTM MP (Amersham Biosciences, UK)
 - Developer (Curix 60, AGFA GEVAERT N.V., Germany)

Procedure:

PVDF membrane and filter papers were cut to cover the gel. In order to rehydrate the membrane, it was soaked in methanol, rinsed in distilled water and transferred to 10 x Towbin buffer. Fibre pads and filter papers were then immersed in 10 x Towbin buffer, and filter pads, filter papers, gel and PVDF membrane were transferred to the cassette and placed in the blotting tank. An ice cooling unit was added to the tank which was filled with 10 x Towbin buffer and set at 100 V for 1 hour. After 1 hour the membrane was dyed with Ponceu solution to compare the protein concentration in the wells. The membrane was then washed in distilled water to remove the Ponceu solution, and in order to saturate the binding sites, the membrane was removed to 3 % Albumine from bovine serum (BSA) in TBS-T and incubated on a rocking platform for 1 hour at room temperature.

Next, primary antibody was diluted in BSA in TBS-T at desired concentration, and the membrane was transferred to the antibody solution and incubated on a rocking platform over night at 4°C. The next morning the membrane was washed 3 x 5 minutes with TBS-T, and then incubated for 45 minutes in a suitable secondary antibody conjugated to horseradish peroxidase (HRP) diluted in TBS-T at the concentration 1:10 000. The membrane was washed 3 x 5 minutes with TBS-T and rinsed in distilled water, ECL solution was prepared and the membrane was incubated with this solution for 5 minutes. The membrane was then transferred to a cassette and developed using HyperfilmTM MP and Curix 60 AGFA developer.

2.7 Immunoprecipitation (IP)

Materials:

- RIPA buffer (as described)
- Id2 (c-20) rabbit polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz; cat.no: Sc-489)
- CTD110.6 monoclonal antibody (Covance, UK; cat.no: MMS-248R)
- Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz; cat.no: Sc-2003)

Procedure:

The plates were placed on ice, and the cell monolayers were washed 3 x in 10 ml ice-cold PBS carefully added to one side of the plate, and removed by suction. After removal of PBS, RIPA buffer was added in various volumes, depending on the desired final concentration of the IP-solutions, to the cell monolayer in order to lyse the cells. The lysates were then scraped of the plate and transferred to ice-cold eppendorf tubes, two plates were mixed in each tube. The tubes were then incubated on ice for 30 min, and insoluble fraction was removed from lysate by centrifugation at 14 000 rpm for 10 min at 4°C. Supernatants were transferred to new, ice-cold eppendorf tubes and 20 µl was removed for protein concentration measurements. Subsequently, the samples, in the presence of 30 µl Protein G Agarose beads and in the absence or presence of Id2 antibody (1:100 dilution) or CTD110.6 antibody (1:2000), were incubated at 4°C over night (Id2) or for 3 hours (CTD110.6). Then the beads were washed 3 x with PBS and resuspended in 30 µl 2 x Loading Dye and boiled for 5 minutes.

2.8 Nuclear extract (NE) preparation

Materials:

- Hanks' Balanced Salt Solution (HBSS) (PAA Laboratories GmbH, Austria; cat.no: H15-009)
- 0.1 % fatty free BSA (Sigma-Aldrich Co, USA; cat.no: A 6003) in HBSS
- PBS (without Ca²⁺ and Mg²⁺) (Gibco; Invitrogen, Norway; cat.no: 10010-015)
- TBS-T (as described)

- Hypotonic buffer

- Tris/HCl (Sigma-Aldrich Co, USA; cat.no: T 1503)
- NaCl (Fluka Chemicals GmbH, Germany; cat.no: 71381)
- MgCl₂ (Sigma-Aldrich Co, USA; cat.no: M 4880)
- ALLN (Calbiochem^R, USA; cat.no: 208719)
- AEBSF (Calbiochem^R, USA; cat.no: 101500)
- Mix of protease inhibitors (Roche Diagnostics, Germany; cat.no: 13181300)

- Dent & Latchman buffer

- Hepes (Sigma-Aldrich Co, USA; cat.no: H 4034)
- Glycerol (Sigma-Aldrich Co, USA; cat.no: G 5516)
- MgCl₂ (Sigma-Aldrich Co, USA; cat.no: M 4880)
- EDTA (Sigma-Aldrich Co, USA; cat.no: E 5134)
- ALLN (Calbiochem^R, USA; cat.no: 208719)
- AEBSF (Calbiochem^R, USA; cat.no: 101500)
- Mix of protease inhibitors (Roche Diagnostics, Germany; cat.no: 13181300)
- DTT (Fermentas, Sweden; cat.no: R 0861)

Procedure:

The medium was removed from each dish (10 cm) and 5 ml HBSS/0.1 % BSA (fatty acid free) was added. The cells were then scraped and two and two parallels were transferred to 15 ml falcon tubes and mixed well by quick vortexing. The cells were then spinned at 320 RCF for 5 min at 4°C. The supernatant was removed, 1 ml PBS was added to each tube and the pellet was dissolved by flicking on the tube. The solutions were transferred to cold 1.5 ml eppendorf tubes and spinned at 320 RCF for 5 min at 4°C. The supernatant was carefully removed and the pellet was resuspended in 450 µl of hypotonic buffer by vortexing. 50 µl 5 % NP-40 was added and immediately, the tubes were flicked on, and the cells were spinned at 120 RCF for 5 min at 4°C. Post nuclear supernatant (PS) was transferred to new, cold eppendorf tubes and stored at -70°C. Nuclear pellet was resuspended in 1 ml hypotonic buffer, (10 µl was removed in order to study the whole nuclei in microscope) and the tubes were spinned at 120 RCF for 5 min at 4°C. The supernatant was removed and 100 µl Dent

and Latchman buffer as well as 5 M NaCl to final concentration of 400 mM was added. The samples were vortexed for 3 seconds and incubated on a roller for 30 min at 4°C. The tubes were then spun at 14000 RPM for 30 min at 4°C, and supernatants were transferred to new, cold eppendorf tubes and stored at -70°C (20 µl of the samples was removed for concentration measurements).

2.9 Immunofluorescence (IF)

Materials:

- PBS (without Ca^{2+} and Mg^{2+}) (Gibco; Invitrogen, Norway; cat.no: 10010-015)
- PBS-T
 - PBS (without Ca^{2+} and Mg^{2+}) (Gibco; Invitrogen, Norway; cat.no: 10010-015)
 - Tween 20 (Sigma-Aldrich Co, USA; cat.no: P 9416)
- Saponin (Fluka Chemicals GmbH, Germany; cat.no: 84510)
- Fixatives (formaldehyde) (Sigma-Aldrich Co, USA; cat.no: F 8775)
- 0.2 % saponin in PBS
- 0.2 % saponin in PBS-T
- PBST-BSA- 0.2 % saponin
- Id2 (c-20) rabbit polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz; cat.no: Sc-489)
- RL2 monoclonal antibody (Snow, 1987 (97))
- OGT/AL28 (Hart, 2003 (45))
- Alexa 488: FITC/Green Rabbit secondary antibody (Molecular Probes, USA; cat.no: A 11008)
- Alexa 546: Red Mouse secondary antibody (Molecular Probes, USA; cat.no: A 11003)
- DAPI (Molecular Probes, USA)
- Dakocytomation fluorescent mounting medium (DAKO) (Dakocytomation, Denmark; cat.no: S 3023)

Procedure:

The cells were cultivated on cover slips in 12 well plates, and after 7 days, media was removed and the cells were washed 3 times in PBS (with no calcium or magnesium). Excess fluid was removed, 100 µl fixatives were added and the cells were incubated for 15 min at room temperature. The lid was placed on to avoid evaporation. Fixatives were removed and the cells were rinsed and washed 2 x 5 min in PBS before permeabilization of the cells in 100 µl 0.2 % saponin in PBS for 15 min at room temperature, with lid placed on. The cells were washed 2 x 5 min in PBST containing 0.2 % saponin, followed by incubation for 15 min in 100 µl blocking buffer containing PBST with 2 % BSA including 0.2 % saponin. Next, primary antibody solutions were made up at desired concentration (usually 1:100) in PBST-BSA-0.2 % saponin. Blocking solution was removed and 40 µl antibody solutions were added onto each sample and incubated for 60 min at room temperature before the cells were washed 3 x 5 min in PBST-BSA-0.2 % saponin followed by incubation in secondary rabbit or mouse Alexa antibody solution at the concentration 1:500 in PBST-BSA-0.2 % saponin for 30 min at room temperature. The lid was placed on and the plate covered by a polystyrene box to avoid fading of fluorescent dyes. The cells were then washed 2 x 5 min with PBST and incubated with DAPI diluted 1:50 000 in PBST in order to stain the nucleus. The slides were mounted with 10 µl antifade mounting media (DAKO) and dried in the dark over night at room temperature.

3. Results

3.1 Effects of hyperglycaemia on Id1 and Id2 protein levels

Previous studies have shown that Id1 protein is regulated by glucose in pancreatic β -cells (77), and Id2 protein in J774.2 macrophages has also been shown to be glucose regulated (79). Glucose-mediated regulation of Id1 and Id2 in primary human macrophages has not yet been studied. Primary human macrophages were treated with 0.5 mM, 5 mM or 20 mM D-glucose for 30 minutes, 60 minutes, 24 hours or 48 hours. Figure 3.1 shows that the relative protein levels of Id1 and Id2 are not affected by hyperglycaemia in primary human macrophages.

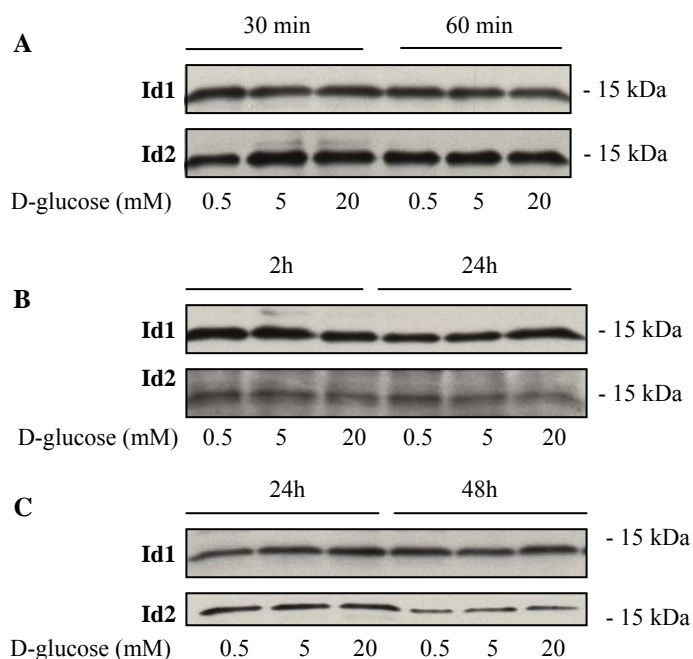


Fig.3.1 Id1 and Id2 protein expression are not affected by hyperglycaemia in primary human macrophages. Primary human macrophages were treated with 0.5, 5 or 20 mM D-glucose for the indicated times. Cells were lysed in RIPA buffer and samples were separated on SDS-PAGE. Id1 and Id2 protein level were determined by Western blotting.

3.2 Effects of hyperglycaemia on Id1 and Id2 protein level in the presence of insulin and/or leptin

In contrast to primary human macrophages, Id2 protein levels are up-regulated by hyperglycaemia in the murine J774.2 macrophage cell line. Human macrophages express insulin receptors and the glucose transporter Glut1. It is currently not known whether insulin via PI3-kinase signalling promote glucose uptake in human macrophages. For this reason, we wanted to investigate whether treatment with insulin or leptin (strong activator of PI3-kinase in macrophages) would affect Id2 and Id1 protein expression in hyperglycaemia treated cells. Furthermore, Id mRNA expression has been shown to increase in response to serum or growth factors, including insulin (77). Primary human macrophages were treated with 5 mM or 20 mM D-glucose in the absence or presence of insulin and/or leptin for 24 hours. Figure 3.2 shows that Id1 is slightly up-regulated by insulin in normoglycaemic conditions, while in hyperglycaemic conditions, insulin and leptin together up-regulate Id1. Figure 3.2 also shows that Id2 is strongly up-regulated by insulin at both normoglycaemic and hyperglycaemic conditions suggesting that high glucose does not affect Id2 protein level in primary human macrophages. Furthermore, under normoglycaemic conditions, leptin up-regulates Id2 protein, in agreement with previously performed micro array experiments in J774.2 cells (data not shown). This suggests that leptin-mediated up-regulation of Id2 protein expression in primary human macrophages occurs at the transcriptional level. All together, these results suggest that Id1 mRNA is up-regulated by insulin, and that both insulin and leptin up-regulate Id2 mRNA in macrophages with no further effect of high glucose.

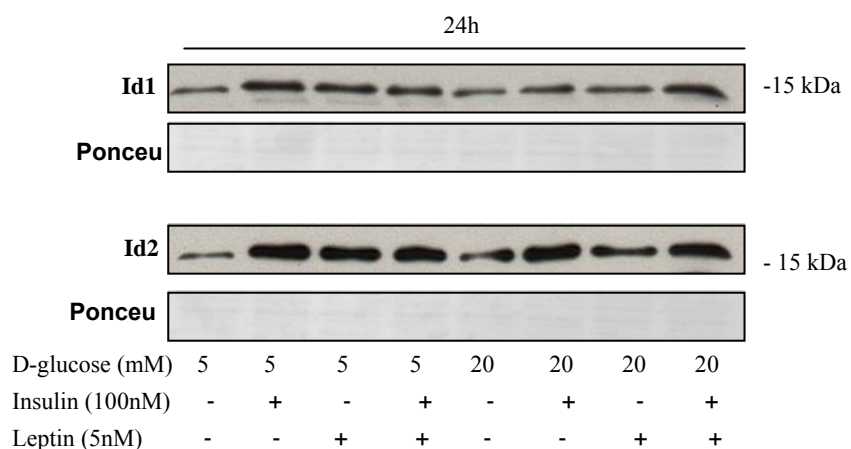


Fig.3.2 Insulin treatment increases Id1 and Id2 protein level in primary human macrophages.

Primary human macrophages were incubated in 0.5, 5 or 20 mM D-glucose for 24 hours in the absence or presence of insulin and/or leptin. Cells were lysed in RIPA buffer and samples were separated on SDS-PAGE. Id1 and Id2 protein level were determined by Western blotting.

Membranes were stained using Ponceu. Representative of two individual experiments performed in duplicate.

3.3 Effects of glucosamine on Id1 and Id2 protein levels

Glucosamine-6-phosphate is formed by the conversion of fructose-6-phosphate by the rate limiting enzyme GFAT, but this molecule can also be produced by the phosphorylation of glucosamine. Glucosamine is transported into the cells via the Glut family of facilitative glucose transporters, and is phosphorylated to glucosamine-6-phosphate. The molecule enters the hexosamine signalling pathway downstream of GFAT, and is able to mimic the effect of glucose acting via this particular pathway (79). To study the effect of glucosamine on the protein levels of Id1 and Id2, primary human macrophages, were treated with 5 mM D-glucose in the absence or presence of 2 mM D-glucosamine or the inhibitor of OGA, PUGNAc, for 4 hours or in the absence or presence of fructose for 4 hours. Figure 3.3 shows that both glucosamine and PUGNAc increases the protein level of both Id1 and Id2 after 4 hours, but that the up-regulation of Id2 are stronger than of Id1.

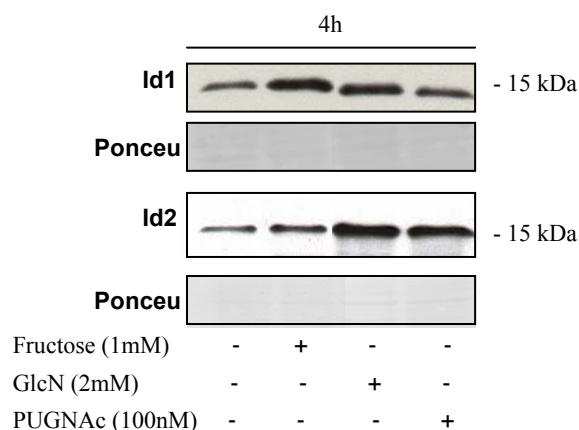


Fig.3.3 Glucosamine and PUGNAc treatment increases level of Id1 and Id2 protein in Primary human macrophages. Primary human macrophages were incubated in 5mM D-glucose in the absence or presence of glucosamine (2mM), fructose (1mM) or PUGNAc. Cells were lysed in RIPA buffer, and samples were separated on SDS-PAGE. Id1 and Id2 protein level were determined by Western blotting. Membranes were stained using Ponceu. Representative of two individual experiments performed in duplicate.

3.4 Effects of fructose on Id1 and Id2 protein levels

During the past several years, consumption of fructose has increased considerably (1). Fructose is absorbed in the small intestine and mostly transported via the portal vein to the liver where it is metabolized by fructokinase to fructose-1-phosphate (89). Fructokinase is not present in extra-hepatic organs, but as serum concentration of fructose increases, fructose is converted to fructose-6-phosphate by hexokinase in extra-hepatic organs (1, 2). The fructose transporter, Glut 5, is strongly up-regulated in macrophages during differentiation, suggesting that these cells become extremely sensitive to small increases in fructose levels (8). To study the effect of fructose on the protein levels of Id1 and Id2, primary human macrophages were treated with 5 mM D-glucose in the absence or presence of fructose for 4 hours. Figure 3.4 shows that fructose strongly up-regulates the protein level of Id1 after 4 hours, and the Id2 protein level is also slightly increased.

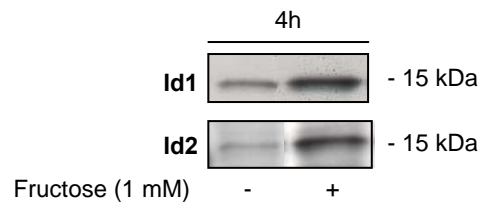


Fig.3.4 Fructose treatment for 4 hours increases level of Id1 and Id2 in primary human macrophages. Primary human macrophages were incubated in 5 mM D-glucose in the absence or presence of fructose (1 mM). Cells were lysed in RIPA buffer and samples were separated by SDS-PAGE. OGT and GFAT protein levels were determined by Western blotting. Representative of two experiments performed in duplicate.

3.5 Identification of O-GlcNAcylated Id2 protein in primary human macrophages

Experiments in J774.2 macrophages suggest that Id2 is target for O-GlcNAcylation (79). Here, we investigated whether Id2 is O-linked glycosylated in primary human macrophages. Cells, cultured under normoglycaemic conditions, were lysed in RIPA buffer and Id2 was immunoprecipitated over night and O-GlcNAcylated Id2 proteins were identified by SDS page and western blotting using CTD 110.6 monoclonal antibody against O-linked glycosylated (O-GlcNAc) proteins (Made specifically against C-terminal domain, a part of RNA polymerase II). In order to detect various O-GlcNAc-modified proteins, immunoprecipitation was performed using the CTD 110.6 monoclonal antibody for 3 hours, and protein levels of O-GlcNAcylated proteins, including Id2 (Figure 3.5A), were determined by SDS page and western blotting using CTD 110.6 monoclonal antibody and Id2 polyclonal antibody. Figure 3.5B shows that Id2 was co-precipitated with the CTD110.6 antibody and also, specific O-GlcNAc immunoreactive bands of approximately 15, 30 and 50 kDa were detected in primary human macrophages.

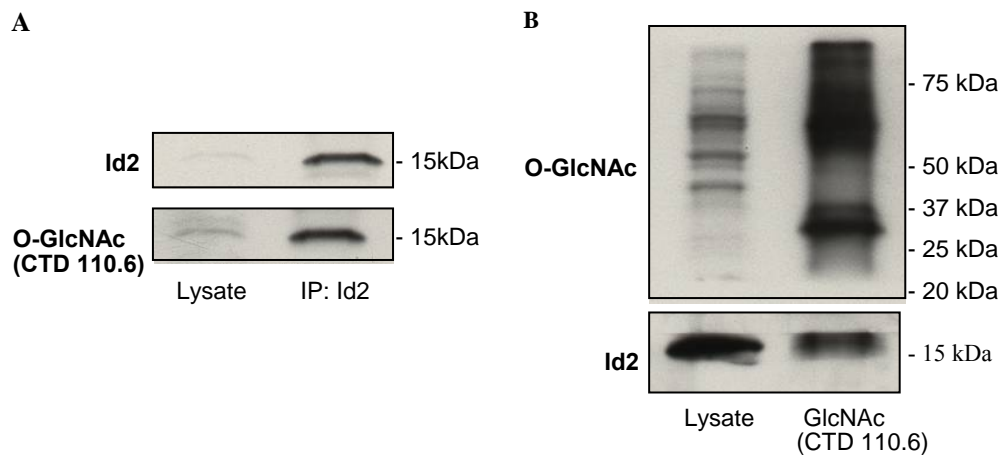


Fig.3.5 Abundance of O-GlcNAylated Id2 protein. Primary human macrophages exposed to normoglycaemic conditions were lysed in RIPA buffer and Id2 was immunoprecipitated. SDS page and Western blotting was performed on whole cell lysates using Id2 polyclonal antibody and CTD 100.6 monoclonal antibody against O-linked glycosylated (O-GlcNAc) proteins. Figure 5 A reveals specific Id2 and O-GlcNAc immunoreactive bands of 15 kDa and figure 5B reveals specific Id2 and O-GlcNAc immunoreactive bands at approximately 15, 30 and 50 kDa.

3.6 Subcellular localization of Id2

Appropriate subcellular localization is crucial for proper function of numerous proteins, and nucleo-cytoplasmic shuttling is a mechanism for regulation of the function of Id proteins (90). Nuclear export has been shown to play an inhibitory role in the suppressive activity of Id2 (90). A way to study the subcellular distribution of proteins is by immunofluorescence. Cells differentiated in 12-wells plates on cover slips were treated with 5 mM and 20 mM D-glucose for 24 hours. Subsequently, the macrophages were treated with Id2 primary antibody diluted 1:50 for 1 hour before further treatment for 30 minutes with secondary Alexa antibody 488, diluted 1:500. In order to stain the nucleus, cells were incubated in DAPI, diluted 1:50 000, and the slides were mounted with DAKO antifade mounting media. Figure 3.6 shows a stronger nuclear staining of Id2 in cells incubated in 5 mM D-glucose compared to 20 mM D-glucose, with slightly stronger cytoplasmic staining in cells treated with high glucose, suggesting active transport of Id2 to the cytoplasm after 24 hours in hyperglycaemic conditions.

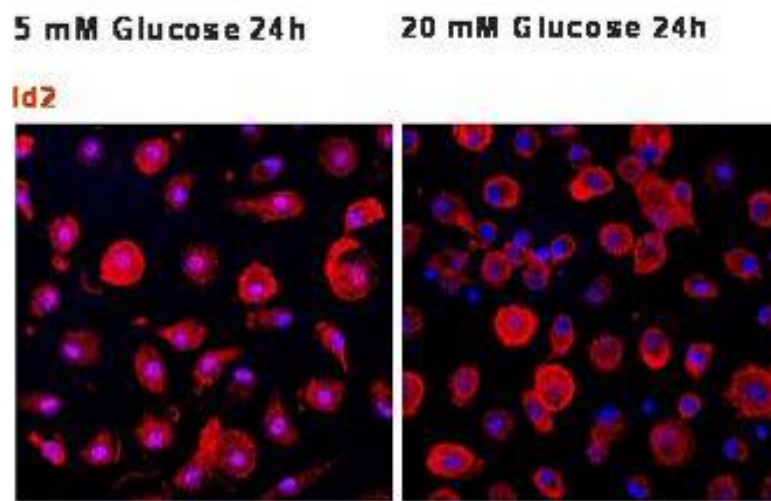


Fig.3.6 Subcellular localization of Id2. Primary human macrophages differentiated in 12-wells plates on cover slips were treated with 5 mM and 20 mM D-glucose for 24 hours followed by treatment with Id2 primary antibody and secondary Alexa antibody 488. Nuclear staining was performed with DAPI.

3.7 Effects of hyperglycaemia on GFAT and OGT protein expression

In order to investigate the regulation of protein levels of GFAT and OGT by glucose, primary human macrophages were incubated in 0.5 mM, 5 mM or 20 mM glucose medium for 30 min, 60 min, 2 h, 24h or 48 h. Figure 3.7 shows that the relative protein levels of GFAT and OGT in primary human macrophages are not affected by hyperglycaemia.

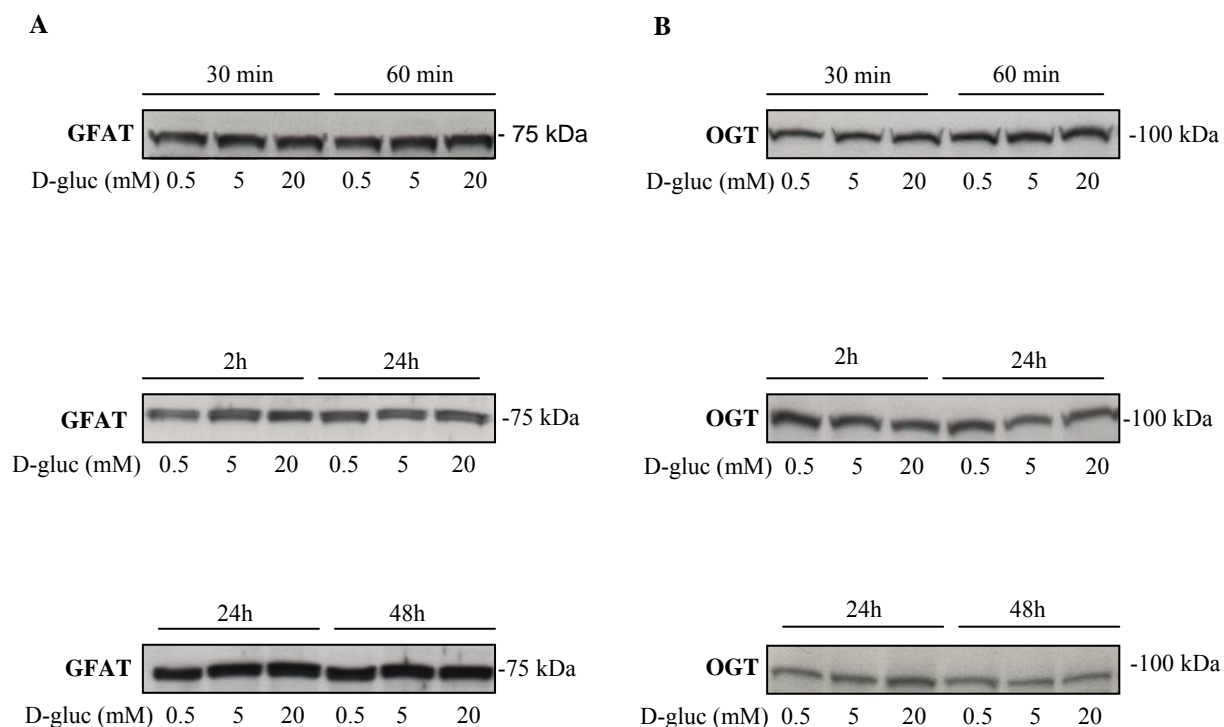


Fig.3.7 GFAT and OGT protein level in primary human macrophages are not affected by hyperglycaemia. Primary human macrophages were treated with 0.5, 5 or 20 mM D-glucose for the indicated times. Cells were lysed in RIPA buffer and samples were separated by SDS-PAGE. OGT and GFAT protein levels were determined by Western blotting.

3.8 Effects of hyperglycaemia in the presence of insulin and/or leptin on GFAT and OGT protein expression

Conditions that characterize type-2 diabetes are hyperglycaemia, hyperleptinemia and hyperinsulinemia. In liver cells, insulin has been shown stimulate the synthesis of OGT (58), suggesting that insulin is able to affect gene transcription of OGT. As macrophage deposition of cholesterol ester has been shown to increase in response to high glucose in the presence of insulin or leptin (81), high glucose is suggested to induce cholesterol ester accumulation in macrophages by affecting insulin and/or leptin signalling. Moreover, insulin and leptin, possibly via PI3-kinase signalling might be able to modulate flux through the hexosamine signalling pathway by regulating GFAT expression and/or activity. Primary human macrophages were treated with 0.5, 5 or 20 mM D-glucose in the absence or presence

of insulin and/or leptin, and the results suggest that the relative protein levels of GFAT and OGT in primary human macrophages are not affected by hyperglycaemia in the presence of insulin and/or leptin (Figure 3.8).

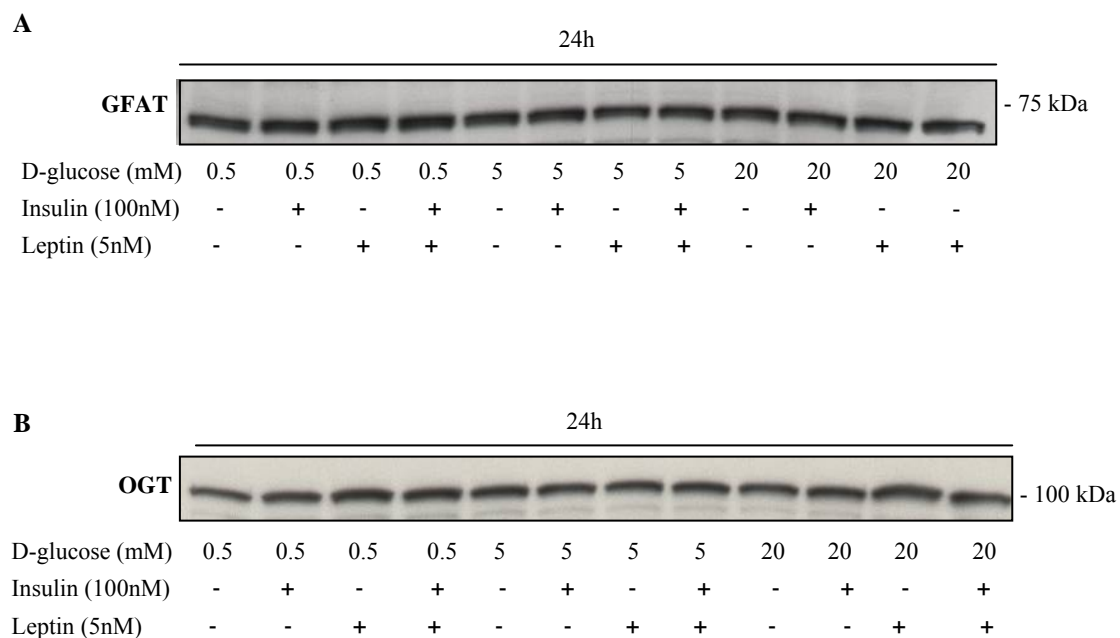


Fig.3.8 GFAT and OGT protein level in primary human macrophages are not affected by insulin and/or leptin. Primary human macrophages were treated with 0.5, 5 or 20 mM D-glucose for 24 hours in the absence or presence of insulin and/or leptin. Cells were lysed in RIPA buffer and samples were separated on SDS-PAGE. OGT and GFAT protein level was determined by Western blotting.

3.9 Effects of glucosamine on the regulation of GFAT and OGT protein expression

In order to investigate the role of the hexosamine signalling pathway in the regulation of the protein levels of GFAT and OGT protein, primary human macrophages were incubated in 5 mM D-glucose medium in the absence or presence of 0,2 mM or 2 mM D-glucosamine for 2 or 6 hours. Western blots show that the relative protein levels of GFAT and OGT in primary human macrophages are not affected by glucosamine (Figure 3.9).

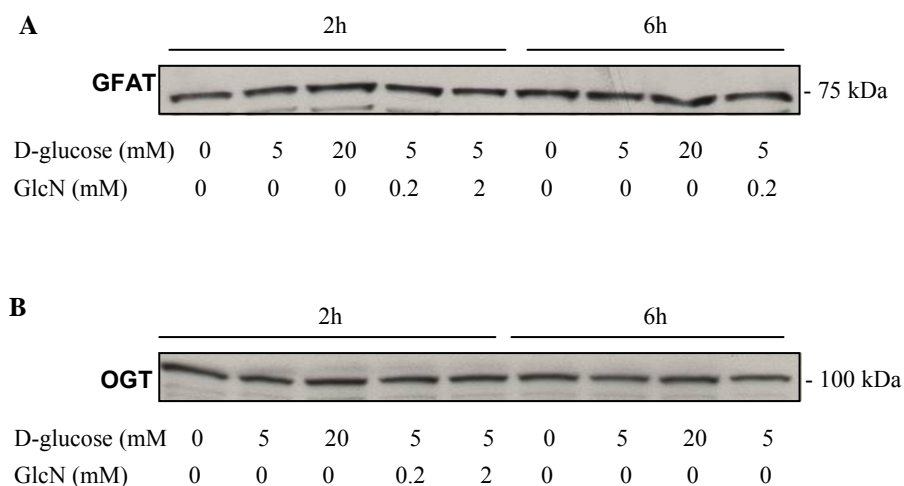


Fig.3.9 Glucosamine treatment does not affect OGT or GFAT protein level in primary human macrophages. Primary human macrophages were treated with 0, 5 or 20 mM D-glucose for 2 or 6 hours in the absence or presence of glucosamine (0.2 or 2mM as indicated). Cells were lysed in RIPA buffer and samples were separated by SDS-PAGE. OGT and GFAT protein levels were determined by Western blotting.

3.10 Effects of fructose on GFAT and OGT protein level

Fructose-6-phosphate is substrate for GFAT in the hexosamine signalling pathway, and in order to investigate regulation of the protein levels of GFAT and OGT by fructose, primary human macrophages were incubated in 5 mM D-glucose medium in the absence or presence of 1 mM fructose. Figure 3.10 shows that the relative protein levels of GFAT and OGT in primary human macrophages are not affected by fructose at 4 hours.

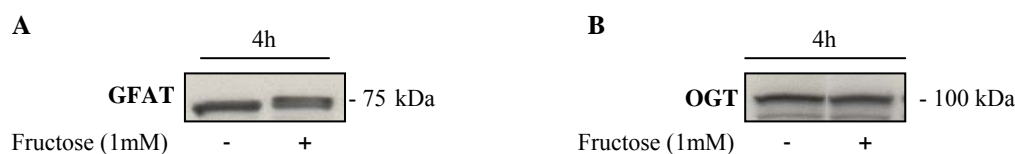


Fig.3.10 GFAT and OGT protein level in Primary human macrophages is not affected by short term stimulation by fructose. Primary human macrophages were treated with fructose (1mM) in 5mM D-glucose for 4 hours. Cells were lysed in RIPA buffer and samples were separated by SDS-PAGE. OGT and GFAT protein levels were determined by Western blotting.

3.11 The effect of hyperglycaemia on the subcellular localization of OGT

OGT enzyme activity and targeting of O-GlcNAc cycling is suggested to be tightly regulated by protein interactions (52, 53, 65, 66,), tyrosine phosphorylation and autoglycosylation (56). This regulation is strongly influenced by environmental factors, and the concentration of UDP-GlcNAc, which increases in response to hyperglycaemia, has been shown to play a key role in regulating the enzymatic activity (35). The enzymatic activity may be due to subcellular localization of this enzyme, and in order to investigate whether the distribution of OGT is regulated by high glucose and/or glucosamine, nuclear extract preparation and immunofluorescence were performed. Primary human macrophages were treated with 5 mM or 20 mM D-glucose or 5 mM D-glucose in the presence of glucosamine (0.2 or 2 mM) or fructose (1mM) and the cells underwent nuclear extract preparation. The relative protein level of the nuclear extracts was determined by SDS page and western blotting. Figure 3.11A shows that nuclear OGT protein levels are up-regulated in response to high glucose, glucosamine and fructose suggesting that high glucose and fructose is able to regulate the localization of OGT protein via the hexosamine signalling pathway.

The immunofluorescence experiment also shows the distribution of OGT and O-GlcNAcylated proteins in primary human macrophages. Cells differentiated in 12-wells plates on cover slips were treated with 5 mM and 20 mM D-glucose for 24 hours. Subsequently, the macrophages were treated with OGT polyclonal antibody or RL2 monoclonal primary antibody detecting O-GlcNAcylated proteins diluted 1:100 for 1 hour before further treatment for 30 minutes with secondary Alexa 488 and 562 antibodies, diluted 1:500. In order to stain the nucleus, the cells were incubated in DAPI, diluted 1:50 000, before the slides were mounted with DAKO antifade mounting media. According to the immunofluorescence experiment, treatment with 20 mM glucose for 24 hours seem to increase the level of OGT protein, and the protein seemed to be localized mainly in the plasma membrane (Figure 3.11B). This is in contrast to previous findings determined by SDS page and western blotting in the current study.

O-GlcNAcylated proteins seem to be localized in the nucleus in both normoglycaemic and hyperglycaemic treated macrophages.

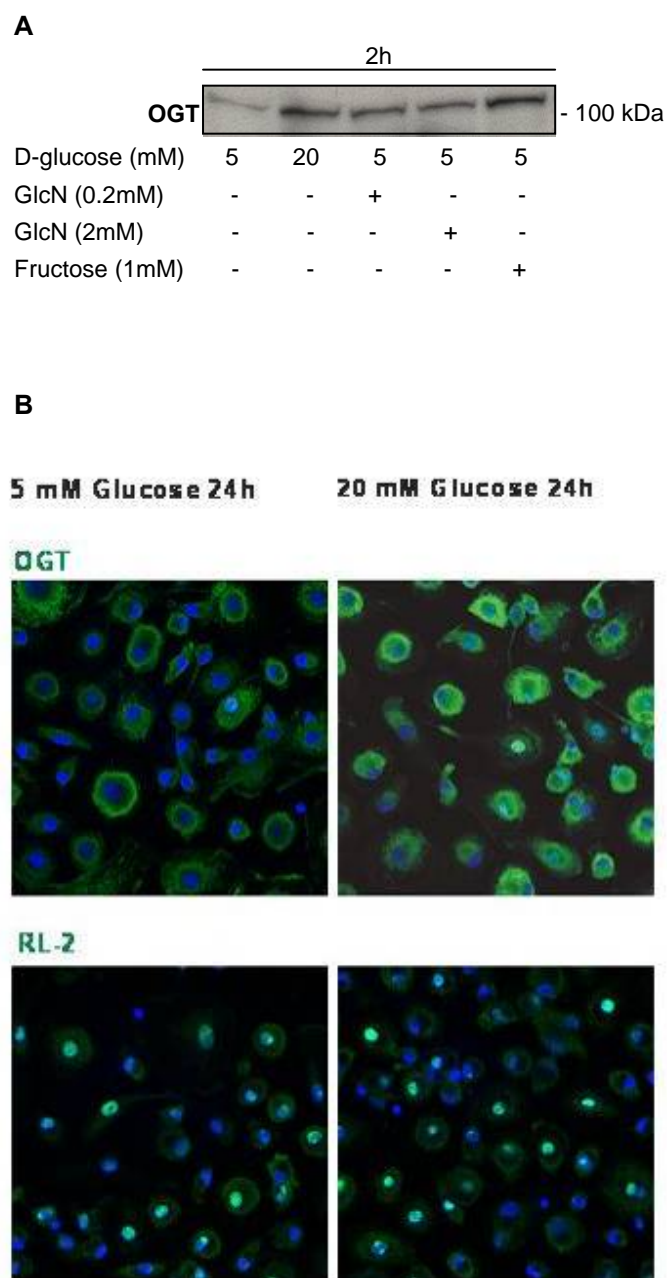


Fig.3.11 Subcellular localization of OGT **A.** Primary human macrophages were treated with 5 or 20 mM D-glucose or 5 mM D-glucose in the presence of glucosamine (0.2 or 2 mM) or fructose and the cells underwent nuclear extract preparation. The relative protein level of the nuclear extracts was determined by SDS page and western blotting.

B. Primary human macrophages differentiated in 12-wells plates on cover slips were treated with 5 mM or 20 mM D-glucose for 24 hours followed by treatment with OGT polyclonal antibody or RL2 monoclonal primary antibody detecting O-GlcNAcylated proteins before treatment with secondary Alexa 488 and 562 antibodies. Nuclear staining was performed with DAPI.

3.12 Identification of *novel* hexosamine-induced O-GlcNAcylated proteins

Fructose-6-phosphate is substrate for GFAT in the hexosamine signalling pathway, and the fructose transporter Glut 5 is strongly up-regulated in macrophages. This makes it very interesting to study whether fructose will increase flux through the hexosamine signalling pathway and increase the abundance of GlcNAcylated proteins in human macrophages. Primary human macrophages were treated with 5 mM D-glucose in the absence or presence of 2 mM D-glucosamine or 1 mM fructose for 4 hours. Relative protein levels were determined by SDS page and detected by western blotting using CTD 110.6 monoclonal antibody against O-linked glycosylated (O-GlcNAc) proteins (A). Competition with 10 mM N-acetylglucosamine (B) reveals specific O-GlcNAc immunoreactive bands of approximately 80 and 250 kDa in the macrophages stimulated with fructose and glucosamine (Figure 3.12).

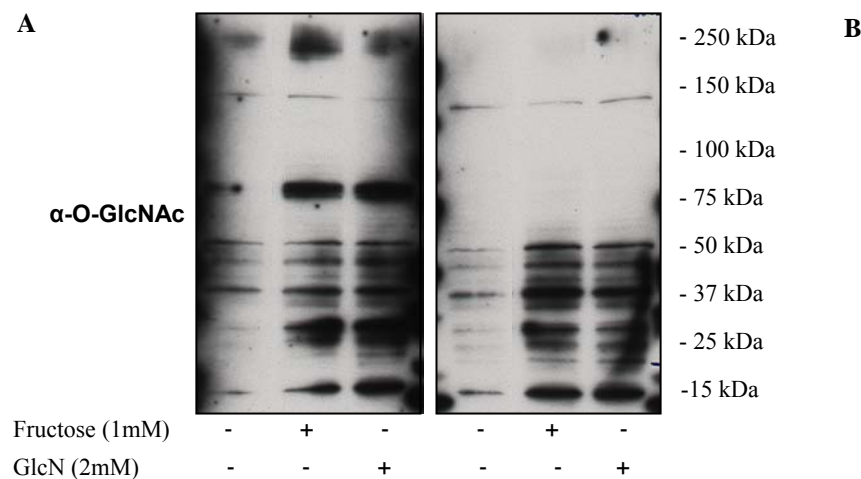


Fig.3.12 Fructose and glucosamine treatment induces O-linked glycosylation on proteins.

Primary human macrophages were treated with fructose (1mM) or GlcN (2mM) in 5mM D-glucose for 4 hours. Cells were lysed in RIPA buffer and SDS page and Western blotting was performed on whole cell lysates using CTD 110.6 monoclonal antibody against O-linked glycosylated (O-GlcNAc) proteins (A). Competition with N-acetylglucosamine (B) reveals specific O-GlcNAc immunoreactive bands of approximately 80 and 250 kDa.

4. Discussion

4.1 Summary of results

In the present study we demonstrate that fructose and glucosamine induce Id1 and Id2 protein expression as well as the abundance of O-GlcNAcylated proteins in primary human macrophages. Moreover, in accordance with previous observations in the J774.2 murine macrophages cell line, Id2 was found to be a target for O-linked glycosylation also in primary human macrophages.

Furthermore, we demonstrated nucleo-cytoplasmic shuttling of Id2 and OGT by high glucose, but in contrast to observations in J774.2 cells, hyperglycaemia has no effect on the protein levels of Id1 and Id2. Finally, hyperglycaemia did not regulate GFAT and OGT protein levels in primary human macrophages.

These data suggest that hyperglycaemia is not able to increase flux through the hexosamine signalling pathway in resting human macrophages as compared to mouse macrophages.

4.2 Id1 and Id2 protein levels are up-regulated via the hexosamine signalling pathway, and Id2 protein is O-GlcNAcylated in primary human macrophages

Previous studies in J774.2 macrophages show that hyperglycaemia increases the protein level of Id2 via the hexosamine signalling pathway (79), and Id1 protein has been shown to be regulated by glucose in pancreatic beta cells (77). The present study in primary human macrophages shows that high glucose has no effect on the protein level of Id1 and Id2. However, both proteins were up-regulated by glucosamine and PUGNAc, suggesting that increased flux through the hexosamine signalling pathway and O-linked glycosylation is involved in the up-regulation of these proteins, also in human macrophages.

Under hyperglycaemic conditions, all cells experience hyperglycemia, but not all cells are affected by it. This depends on the glucose transporter expressed and whether or not the cells increase the uptake of glucose in response to hyperglycaemic conditions. Very few studies

have addressed the effect of hyperglycaemia on human macrophages, and according to this particular study we suggest that these cells are less sensitive to high glucose levels than mouse macrophages. In J774.2 cells, Id2 protein is up-regulated by hyperglycaemia via the hexosamine signalling pathway. We were able to reproduce the up-regulation of Id2 protein via increased flux through the hexosamine signalling pathway in primary human macrophages. However, any effects of hyperglycaemia were not observed, suggesting that flux through the hexosamine signalling pathway, under hyperglycaemic conditions, is not increased in human macrophages compared to murine cells. Hyperglycaemic damage is thought to be mediated by four different pathways, and in one of these pathways, the polyol pathway, the enzyme aldose reductase catalyzes the reduction of glucose-6-phosphate to sorbitol using (NADPH) as cofactor (11, 12). The opposing results of the regulation of Id2 protein level in the current study compared to experiments in J774.2 macrophages, may be explained by the fact that murine macrophages express lower levels of aldose reductase compared to the human cells causing increased flux of glucose through alternative metabolic pathways downstream of glucose-6-phosphate, such as the hexosamine signalling pathway using fructose-6-phosphate and glutamine as substrates. This difference in intracellular glucose utilization may result in increased flux through the hexosamine signalling pathway in J774.2 macrophages compared to primary human macrophages.

Furthermore, superoxide production by phagocytes is associated with a rapid increase in metabolism known as the ‘respiratory burst’, characterized by increased hexose monophosphate shunt (HMPS) activity, which produces NADPH, the substrate required by NADPH oxidase for the production of superoxide (91). This process demands increased sources of glucose for NADPH production, and this may cause decreased hexosamine signalling pathway flux in macrophages.

Appropriate subcellular localization is crucial for the proper function of numerous proteins (90). Large proteins shuttle between the nucleus and cytoplasm through nuclear pore complexes by virtue of their intrinsic nuclear localization signals (NLSs) and nuclear export signals (NESs). In contrast, small proteins with molecular masses of less than 40 kDa can freely pass through the nuclear membrane (92, 93). Nucleo-cytoplasmic shuttling appears to be a mechanism for regulation of the function of Id proteins. Nuclear export has been shown to play an inhibitory role in the suppressive activity of Id2 protein (90). Id proteins have molecular masses ranging from 13-18 kDa, thus Id2 protein has been shown to enter the

nucleus by passive diffusion, and nuclear export and cytoplasmic localization are dependent a NES in the C-terminal region (90). This nucleo-cytoplasmic shuttling of Id proteins is subject to diverse types of regulation. The subcellular localization of many proteins is regulated by protein modifications such as phosphorylation, and in this particular study, Id2 was shown to be a target for O-GlcNAcylation. Using the YinOYang O-linked glycosylation Prediction Server (94), we have found a potential O-GlcNAcylation site at position 108 in the Id2 gene (Figure 4.1) suggesting a role for O-GlcNAcylation in the regulation of nucleo-cytoplasmic shuttling of Id2. In future experiments, the effect of hyperglycaemia on the rate of O-GlcNAcylated Id2 will be addressed as well as the role of O-GlcNAcylated Id2 in macrophage foam cell formation.

In the present study, increased abundance of Id2 protein is observed in the cytoplasm in cells incubated in 20 mM D-glucose compared to 5mM D-glucose at 24 hours. This may be due to activation of nuclear export and a potential cytoplasmic association of Id2 and E proteins/bHLH transcription factors in the cytoplasm under hyperglycemic conditions. This may inhibit the transport of these transcription factors into the nucleus when dimerized to Id2. In future experiments, effects of hyperglycaemia on the subcellular distribution of Id2 will be investigated in a time-dependent manner, and the effect of glucosamine and fructose will also be elucidated.

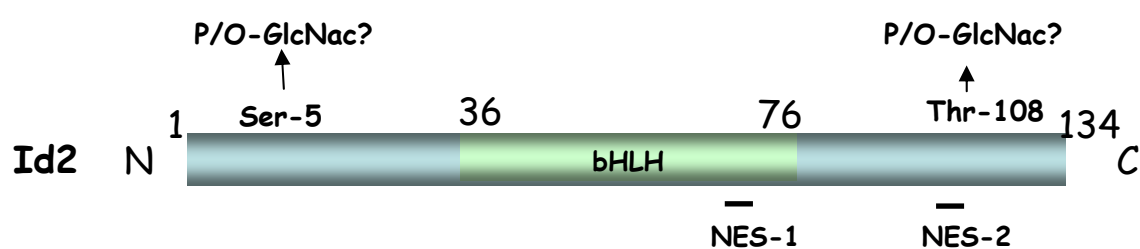


Fig.4.1 O-GlcNAcylation site in the Id2. A potential O-GlcNAcylation site at position 108 in the Id2 gene suggesting modulation of NES-2 and involvement of O-GlcNAcylation in the regulation of nucleo-cytoplasmic shuttling of Id proteins.

In addition to hyperglycaemia, hyperleptinemia and hyperinsulinemia, type-2 diabetes, insulin resistance, and obesity are associated with inflammation and production of pro-inflammatory cytokines such as IL-12 and tumor necrosis factor alfa (TNF-alfa), as well as activation of T-cells. In vivo, glucose ingestion has been shown to induce increases in the pro-inflammatory transcription factor, NF-κB, and increases in TNF-α mRNA (86). In the

pathogenic environment of diabetes, cytokines and T-cells are likely to promote effects of high glucose in macrophages as LPS-activated murine macrophages have been shown to increase the uptake of glucose via up-regulation of Glut1 (95). Future investigations will include studies of hyperglycaemia in activated, human macrophages.

4.3 GFAT protein expression is not regulated by high glucose via the hexosamine signalling pathway in primary human macrophages

The protein level of GFAT in primary human macrophages has previously not been studied, but previous experiments in skeletal muscle indicate up-regulation of GFAT protein level at high concentration of saturated fatty acids. Moreover, experiments in a breast cancer cell line showed that GFAT protein level was up-regulated by epidermal growth factor (EGF) and glucosamine, while high glucose (25 mM) blocked the transcriptional effect of EGF on the GFAT gene (38). The results in this study in primary human macrophages, indicates that GFAT protein level is not regulated by high glucose, most likely because resting human macrophages do not experience glucose toxicity in response to high external glucose levels. Furthermore, the production of superoxide in macrophages may reduce the flux through the hexosamine signalling pathway and make this pathway less sensitive for increased glucose concentrations.

4.4 Subcellular distribution of OGT, but not protein level, is regulated by high glucose via the hexosamine signalling pathway in primary human macrophages

In liver cells, insulin has been shown to stimulate the synthesis of OGT and to enhance cytosolic staining of OGT (58). In the present study, western blotting experiments showed that the protein level of OGT in primary human macrophages was not affected by high glucose via the hexosamine signalling pathway. In contrast, immunofluorescence experiments revealed that treatment with 20 mM glucose for 24 hours appeared to increase the level of OGT protein as determined by stronger OGT staining in hyperglycaemic cells. The antibody used for this studies, AL28, obtained from Gerald Harts lab, has not been affinity-purified, and it has to our knowledge, not been used in immunofluorescence studies before. In future experiments, several different OGT antibodies will be tested.

OGT is subject for autoglycosylation and tyrosine phosphorylation, modulating enzyme activity, substrate recognition and localization (56). The concentration of UDP-GlcNAc, which increases in response to hyperglycaemia, has been shown to play a key role in regulating the enzyme activity (35). Western blotting experiments on nuclear extracts performed in this study indicates that nuclear expression of OGT protein is increased in high glucose treated primary human macrophages suggesting hyperglycaemia-mediated nuclear translocation of OGT in these cells.

According to immunofluorescence experiments, using RL2 monoclonal antibody, O-GlcNAcylated proteins in human macrophages seem to be localized in the nucleus in the cells treated with 5 mM and 20 mM D-glucose for 24 hours. This is in agreement with experiments that showed that staining of 4-cell embryos with RL2 reveals heavy nuclear staining (96). The abundance of O-GlcNAcylated proteins was expected to increase in response to hyperglycaemia, but this was not seen in this specific experiment. The antibody used, RL2, was made specifically against the epitope of nuclear pore (NP62) protein (97), and in future immunofluorescence experiments we will also use the CTD110.6 antibody as Alexa IgM secondary antibodies are now commercially available.

4.5 Fructose up-regulates Id1 and Id2 protein level in primary human macrophages

Consumption of fructose, primarily from fructose containing beverages, has increased considerably during the past several years (1). The main pathway for fructose metabolism is in the liver, due to the presence of the enzyme fructokinase (1). As serum concentration of fructose increases, due to the inability of the liver to metabolize the load completely, fructose is converted to fructose-6-phosphate by hexokinase in extra-hepatic organs (1, 2). The fructose transporter, Glut 5, is strongly up-regulated in macrophages during differentiation (Figure 4.2), and macrophages might become extremely sensitive for small increases in circulating fructose levels (8). In this particular study, fructose appears to up-regulate the protein levels of Id1 and Id2 and we suggest that fructose enters the hexosamine signalling pathway in human macrophages in response to small increases in circulating serum fructose concentrations.

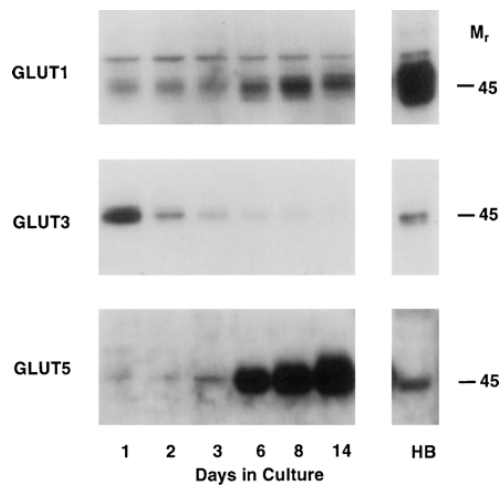


Fig. 4.2 Glut 5 in human macrophages is strongly up-regulated during differentiation Immunoblots of GLUT-1, -3, and -5 expressed in human cultured monocyte-derived macrophages over a 14-day differentiation period. A sample of membranes prepared from human brain (HB; *right lane*) was also blotted as an internal control. M_r , relative molecular weight. (Figure is adapted from Malide D *et al.*, 1998 (8))

4.6 Identification of *novel* hexosamine-induced O-GlcNAcylated proteins

According to the present study, fructose increases the abundance of O-GlcNAcylated proteins, suggesting that fructose increase flux through the hexosamine signalling pathway. These observations are supported by the involvement of the hexosamine signalling pathway in the desensitizing of the glucose transport system (32), and the ability of both glucosamine and fructose to induce insulin resistance, according to previous studies (98, 99, 100, 101). In one study, supplementing the diet of healthy males with 3 g fructose per kg of body weight per day, which increased total caloric intake by 25 percent, resulted in insulin resistance in only six days (98).

Like the increased consumption of fructose during the past several years, glucosamine supplementation has increased considerably. This is primarily due to the belief that glucosamine is efficacious in improving symptoms and joint space narrowing of osteoarthritis of the knee (102, 103, 104, 105). However, one recent intervention trial did not show any significant benefits (106), but as glucosamine is available and relatively inexpensive, it is subject for widespread consumption. As glucosamine is classified as a nutritional supplement, it is subject to minimal regulation, and its effects on other chronic diseases as well as metabolic outcomes have not been well studied (107). As earlier mentioned, glucosamine consumption can result in insulin resistance, and another area of concern is its potential effect on lipid metabolism. In LDL receptor deficient male mice glucosamine consumption exacerbated hyperlipidemia (107). As mention earlier, superoxide

production by phagocytes depends on increased HMPS activity, and may cause decreases in the entering of glucose into the hexosamine signalling pathway in macrophages. Both fructose and glucosamine enter this pathway down-stream for glucose-6-phosphate, and are likely to be important sources for O-GlcNAcylation of proteins in primary human macrophages and potential promoters of macrophage foam cell formation.

4.7 Methodological considerations

In vitro studies in primary human macrophages may be confounded by genetic and physiological differences of the blood donors and profoundly influence the parameters under investigation. However, during the differentiation process that lasts for 7 days, cells are experiencing the same culture conditions, and this is likely to minimize the differences between cell populations. In future studies, for simplification, we will perform initial experiments in the THP-1 human macrophage cell line. Relevant, reproducible observations will then be verified in primary human macrophages.

4.8 Further investigations

One aspect that has not been investigated in the present study, is whether glucose uptake in macrophages is affected by conditions that characterize type 2 diabetes, insulin resistance and obesity; hyperglycaemia, hyperinsulinemia and hyperleptinemia. In macrophages, the mechanism of glucose uptake appears to be facilitated diffusion (108). Glucose is transported via facilitative glucose transporters, and during differentiation to macrophages, Glut 1 increases progressively, Glut 3 decreases rapidly and Glut 5 (which main function is as a fructose transporter) is strongly up-regulated (8). Phagocytosis is associated with dramatic changes in the metabolism of phagocytes (109), and the increased glucose uptake appears to be linked to changes in the carrier mediated transport system (108). In order to investigate whether hyperglycaemia alone or in the presence of insulin or leptin as well as fructose and glucosamine at normoglycaemia, affects glucose uptake in macrophages, further experiments will include glucose uptake assays as well as analysis of protein levels of Glut 1, 3 and 5.

5. Conclusion

In resting human macrophages, the hexosamine signalling pathway is involved in the up-regulation of Id1 and Id2 protein, but hyperglycaemia is not able to increase flux through the hexosamine signalling pathway as compared to murine macrophages and does not regulate GFAT and OGT protein levels.

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Appendix 1

Ingredients	Final concentration
Glycerol	4 %
SDS	0.8 %
DTT	40 mM
Bromophenol blue	0.025 %
Tris buffer, pH 6.8	40 mM

Table 1 1 x Loading buffer.

Ingredients	Final concentration
Tris/HCl, pH 7.0	50 mM
NaCl	150 mM
SDS	0.1 %
NP-40	1 %
Na-Deoxycholate	1 %
EDTA	2 mM
Tx-100	1 %
ALLN	30 μ M
AEBSF	1 mM
Protease inhibitors	1 tablet
DTT	1 mM
NaF	50 mM
Na ₄ PO ₂ O ₇	10 mM
N ₃ VO ₄	1-2 mM

Table 2 1 x RIPA buffer

Ingredients	Final concentration
Tris/HCl	25 mM
Glycine	0.2 M
SDS	0,1 %

Table 3 1 x Running buffer

Ingredients	Final concentration
Tris/HCl	2.5 mM
Glycine	20 mM
Methanol	2 %

Table 4 1 x Towbin buffer

Ingredients	Final concentration
Tris/HCl	10 mM
NaCl	150 mM
Tween 20	0.05 %

Table 5 1 x TBS-T

Ingredients	Final concentration
Tris, pH 7.6	100 mM
NaCl	100 mM
MgCl ₂	30 mM
ALLN	300 µM
AEBSF	25 µM
Protease inhibitors	1 tablet

Table 6 Hypotonic buffer

Ingredients	Final concentration
Hepes, pH 7.9	5 mM
Glycerol	26 %
MgCl ₂	1.5 mM
EDTA	0.2 mM
ALLN	3 µM
AEBSF	250 µM
Protease inhibitors	1 tablet
DTT	200 µM

Table 7 Dent & Latchman buffer